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Contents

Effect of Previous Starvation on the Response of Plasma Lipids and Free Fatty Acids to a Fat Meal. Margaret J. Albrink and Robert S. Neuwirth 441

Suppression of Uric Acid Synthesis in the Gouty Human by the Use of 6-Diazo-5-oxo-L-norleucine. Arthur I. Crayzel, J. E. Seegmiller and Ethel Love 447

Serum Lipid and Lipoprotein Alterations in Nephrosis. James H. Baxter, Howard C. Goodman and Richard J. Havel 455

Evaluation of the Slope-Volume Method as an Index of Pulmonary Blood Volume. Yang Wang, John T. Shepherd and Robert J. Marshall 466

Chromatographic Properties of Gamma Globulin: Behavior of Serum Gamma Macroglobulins. Joseph Lospalluto, Julio Chegoriansky, Arthur Lewis and Morris Ziff 473

A Study of Distal Renal Tubular Functions by a Modified Stop Flow Technique. John R. Jaenike and Robert W. Berliner 481

Bilateral Studies of Cerebral Oxygen Uptake in Young and Aged Normal Subjects and in Patients with Organic Dementia. N. A. Lassen, I. Feinberg and M. H. Lane 491

Protein-Binding of Inorganic Phosphate in Plasma of Normal Subjects and Patients with Renal Disease. Mackenzie Walser 501

The Physiological Significance of the Secretion of Endogenous Insulin into the Portal Circulation. III. Evidence for a Direct Immediate Effect of Insulin on the Balance of Glucose Across the Liver. Leonard L. Madison, Burton Combes, Reuben Adams and William Strickland 507

The Metabolic Effects of the Acetic and Propionic Acid Analogs of Thyroxine and Triiodothyronine. S. Richardson Hill, Jr., S. B. Barker, Jean H. McNeil, John O. Tingley and Lester L. Hibbett 523

Clinical, Morphological and Biochemical Studies of a Virilizing Tumor in the Testis. Kenneth Savard, Ralph I. Dorfman, Billy Baggett, Linda L. Fielding, Lewis L. Engel, Harry T. McPherson, Leonard M. Lister, David S. Johnson, Edwin C. Hamblen and Frank L. Engel 534

Disappearance Rate of Concentrated Proconvertin Extracts in Congenital and Acquired Hypoproconvertinemia. M. Silvija Hoag, Paul M. Aggeler and Alfred H. Fowell 554

Announcement of Meetings 454

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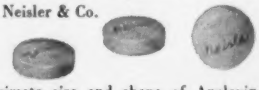

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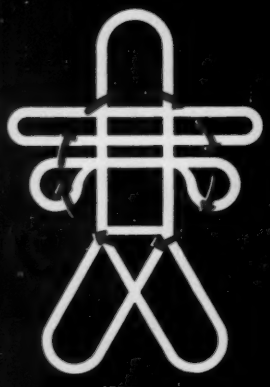

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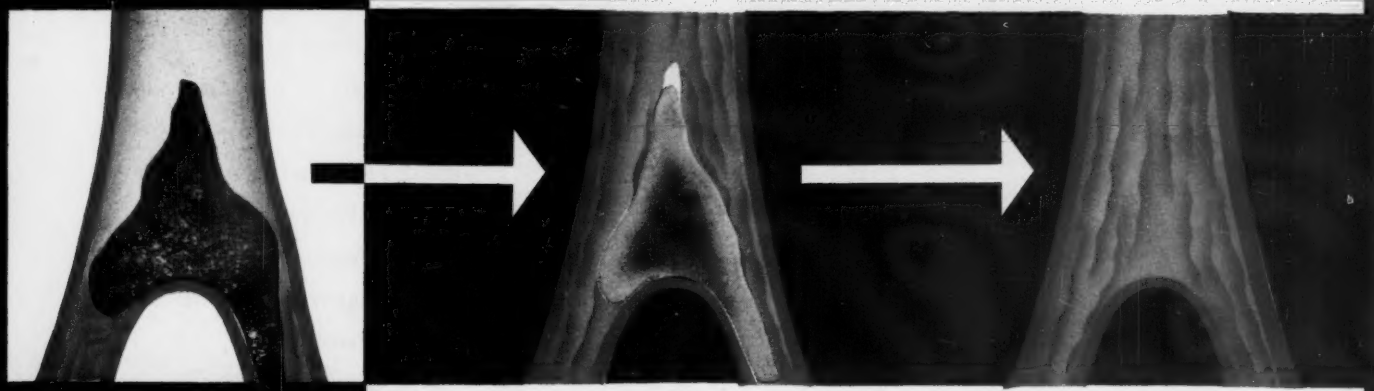

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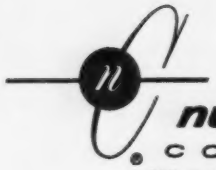

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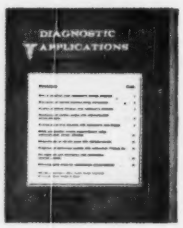

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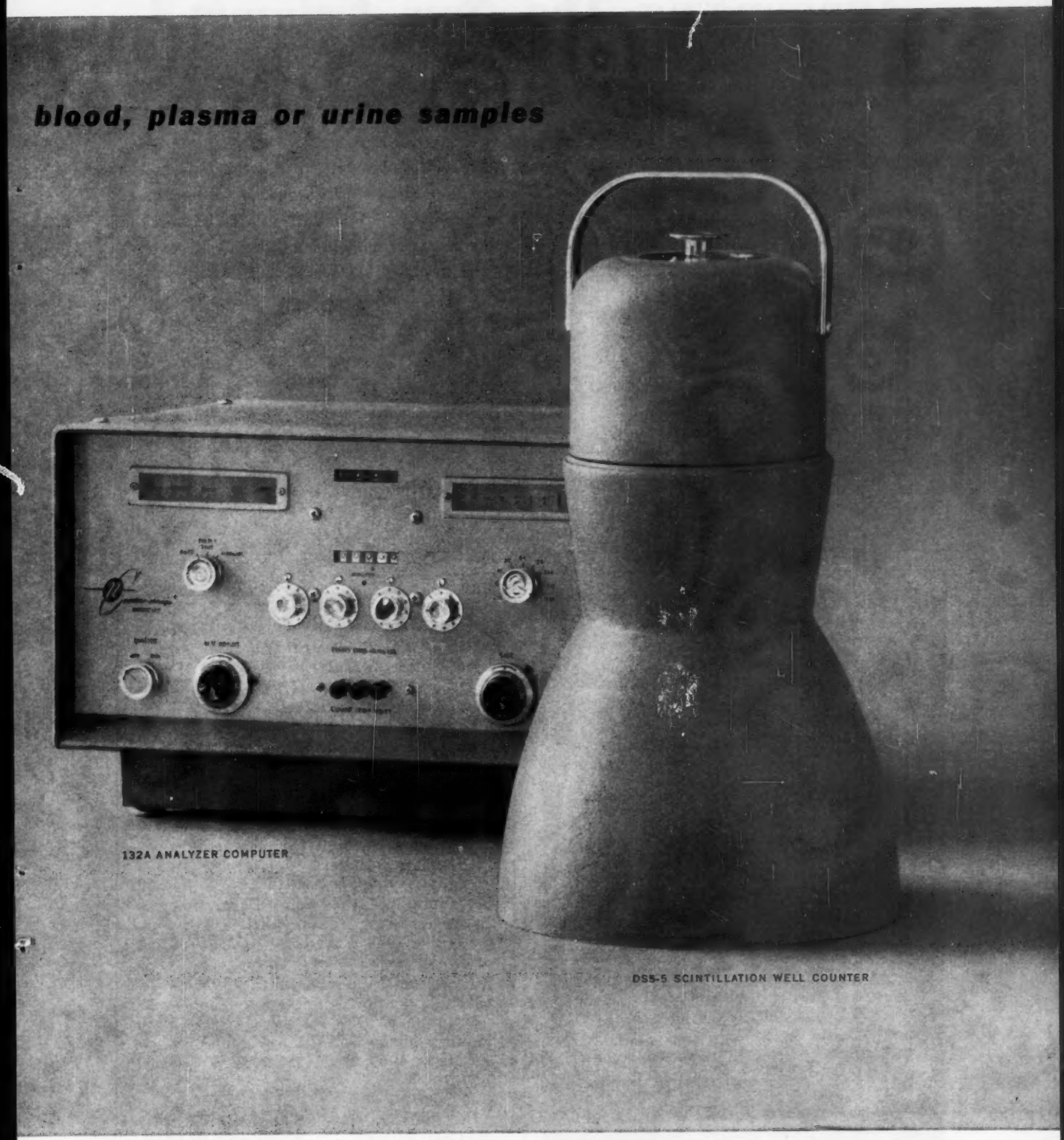

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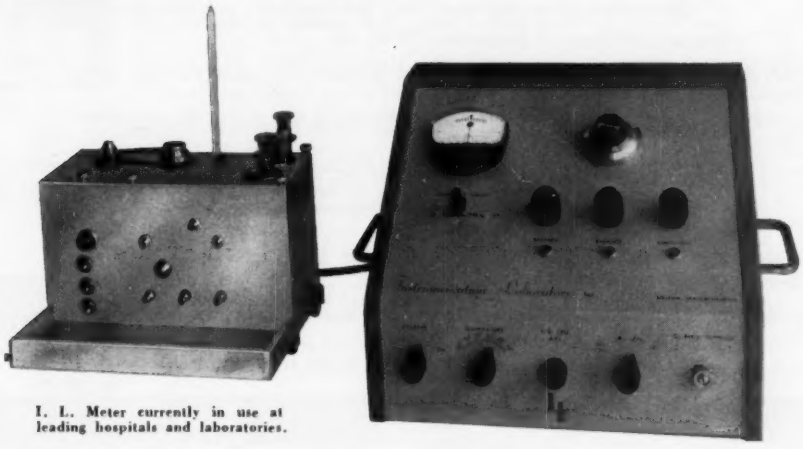

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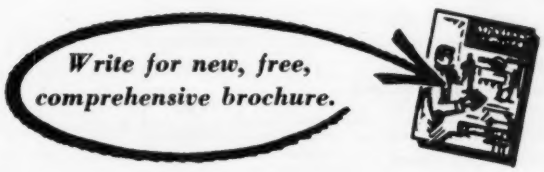

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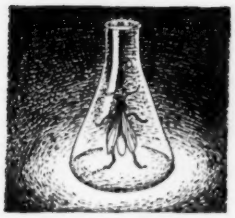

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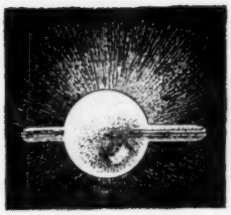

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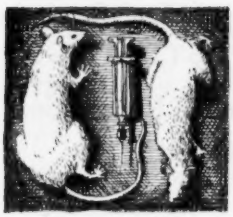

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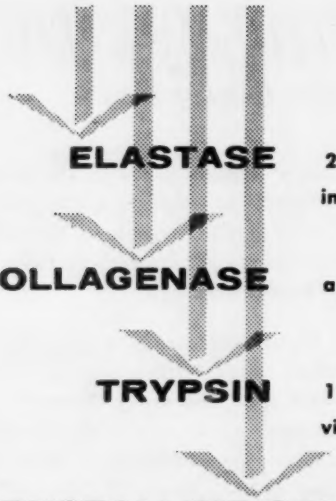

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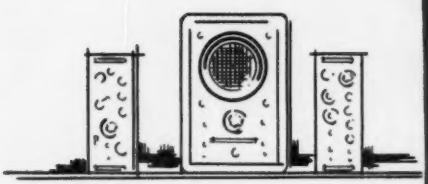

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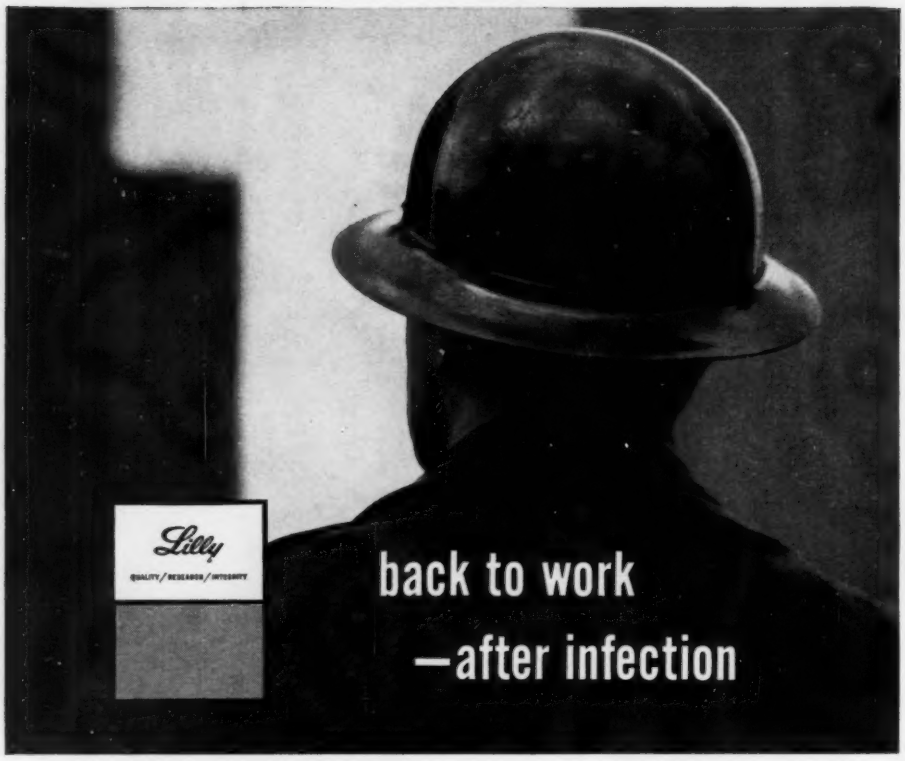

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EFFECT OF PREVIOUS STARVATION ON THE RESPONSE OF PLASMA LIPIDS AND FREE FATTY ACIDS TO A FAT MEAL*

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(Submitted for publication July 30, 1959; accepted August 20, 1959)

Plasma free fatty acids (also variously referred to as nonesterified fatty acids and unesterified fatty acids) have assumed importance as a small but very rapidly metabolized component of serum lipids (1, 2). Their derivation from the fat of adipose tissue has been demonstrated and may represent an important form in which stored fat is mobilized, transported and made available for fuel during starvation (3).

The concentration of free fatty acids (FFA) in plasma increases during starvation (4), in the "metabolic" starvation of diabetic acidosis (5) and of hyperthyroidism (6). When an overnight fast is terminated by the administration of 50 to 100 g of carbohydrate, an abrupt fall in concentration of plasma FFA occurs within one-half hour and lasts for several hours, and a similar but less marked drop follows a protein meal (1, 2). On the other hand, following a fat meal of equal or greater caloric value, the FFA either do not change appreciably or rise much as they do during a similar period of fasting (2, 7).

The decrease in concentration of FFA appears to occur in response to an increase in carbohydrate utilization. The absence of such a drop in concentration after a fat meal would seem to indicate insensitivity of the FFA regulatory system to fat or merely the substitution of FFA derived from ingested fat for those derived from adipose fat (3). An alternative possibility is herein suggested, namely that relative inefficiency of fat metabolism in persons adapted to a high carbohydrate diet might account for the failure of a fat meal to cut off the FFA-mobilizing mechanism. Any nutritional or metabolic maneuvers aimed at increasing fat combustion might alter the response of plasma FFA to a fat meal.

The present study is concerned with the change in plasma lipids including FFA when a fat meal was administered at the end of a period of starvation lasting for several days.

MATERIALS AND METHODS

The subjects, who were in their twenties and were free of known disease, were 6 males, and 2 females. A third woman became ill from the effects of the first 24 hours of starvation and had to be dropped from the study. To 6 of the subjects a fat breakfast was given after an overnight fast, and again at the end of a period of starvation lasting from 2 to 7 days. During the starvation nothing but water was ingested. The subjects carried on their usual activities despite the discomforts of starvation.

The breakfast given to 5 of the 6 subjects receiving the fat meal consisted of 2 eggs, 28 g butter, and 60 ml heavy cream scrambled together. This meal supplies 60 g of fat, 12 g protein, and no carbohydrate. The sixth subject (B. B.) was given the same meal with the difference that the egg white was removed, thus eliminating the small amount of protein present in the breakfast.

In order to compare the effects of the fat breakfast with the effects of a carbohydrate meal, two additional male subjects were fed, after an overnight fast, 100 g of glucose dissolved in 400 ml of water flavored with lemon juice. The same amount of glucose was again administered after 3 days of starvation.

At intervals before and after the test meals and in some instances during the starvation, blood was drawn from an antecubital vein in a heparinized syringe, immediately centrifuged at room temperature, and the plasma separated from the cells. An aliquot for FFA determination was precipitated in the extraction mixture within one-half hour of the venipuncture.

FFA were determined by the Method of Dole (2), using a modified method of standardizing the alkali (8). Total fatty acids (9), total cholesterol (10) and lipid

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† This work was done during tenure of an Established Investigatorship of the American Heart Association.

‡ Some of the data in this article represent work done in fulfillment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

TABLE I

Changes in plasma lipids following a fat meal given after an overnight fast (control) and terminating a period of several days' starvation

Subject Sex		Time after Bk*	Cholesterol	Lip. P	Triglyceride fatty acids	FFA
		hrs	*mg/100 ml*	*mg/100 ml*	*mEq/L*	*mEq/L*
A. M. M	Control	Fasting	216	10.5	2.3	0.38
		3	202	10.4	7.9	0.47
		5	197	11.0	10.0	0.64
	Refeeding on 4th day of starvation	Fasting	219	9.9	5.5	1.40
		3	240	9.2	9.7	0.79
		5	213	11.3	6.7	0.63
	Repeat 1 wk later	Fasting	194	9.5	3.0	0.50
		3	218	11.0	6.0	0.40
		5	211	10.3	6.0	0.55
B. B. M	Control	Fasting	202	9.2	3.4	0.38
		3	190	8.3	4.4	0.55
		5	193	9.7	5.0	0.75
	Refeeding on 4th day of starvation	Fasting	250	10.0	5.8	2.63
		3	245	10.8	6.3	1.74
		5	243	10.7	6.1	1.79
R. N. M	Control	Fasting	238	7.8	6.7	0.41
		3	257	8.3	6.6	0.48
	Refeeding on 8th day of starvation	Fasting	275	9.5	6.4	2.25
		3	254	10.1	8.2	1.71
S. H. M	Control	Fasting	256	9.7	3.2	0.25
		3	253	11.3	5.3	0.42
		5	247	9.2	6.0	0.65
	Refeeding on 3rd day of starvation	Fasting	239	9.7	4.5	2.01
		3	244	10.2	4.7	1.26
		5	235	10.3	3.1	1.28
J. G. F	Control	Fasting	186	8.1	2.3	0.50
		3	193	8.5	4.6	0.62
		5	185	9.4	2.9	0.69
	Refeeding on 3rd day of starvation	Fasting	182	7.2	3.2	0.96
		3	180	8.6	2.6	1.07
		5	189	8.9	2.9	1.18
M. D. F	Control	Fasting	205	9.7	1.7	0.45
		3	217	11.1	2.8	0.57
		5	214	10.9	2.7	0.57
	Refeeding on 4th day of starvation	Fasting	252	11.9	5.6	2.12
		3	250	13.9	6.3	1.25
		5	255	12.9	6.4	1.13

* Bk = breakfast containing 60 g of fat but no carbohydrate.

phosphorus (11) were determined on a chloroform-methanol extract. Triglyceride concentration was estimated by subtracting the fatty acids estimated to be combined with cholesterol and phospholipid from the total fatty acids. Blood glucose (12) was determined in the subjects receiving glucose.

RESULTS

Response to fat meal. The results are listed in Table I. Three and five hours following the control breakfast, the plasma FFA concentration increased in all six subjects, and triglyceride concentration increased in five of the six subjects. Plasma cholesterol and phospholipid concentrations did not change consistently.

In three subjects studied during the course of starvation, two of whom are represented in Figures 1 and 2, a rise in plasma FFA during the first day of starvation was indistinguishable from the changes after the fat meal. With continuation of starvation there was a definite but not consistent afternoon rise, with a drop occurring by the next morning. The overall trend was that of a progressive increase in plasma FFA concentration.

On the final morning of starvation, the FFA had increased in the plasma of all subjects from an initial average concentration of 0.39 mEq per L on the control morning to an average of 1.89 mEq per L (Tables I and II, Figures 1 and 2). The highest concentration of 2.7 mEq per L was achieved in the subject who had fasted a week (R. N.). An increase in cholesterol concentration occurred in the plasma of only three of the subjects, and in the concentration of triglycerides in five. The increases in triglyceride concentration were less than 5 mEq per L, and might in part represent FFA partially included in the chloroform-methanol extract.

The fat meal which terminated the fast produced an opposite effect on plasma FFA concentration from that of the control meal in five of the six subjects: in all but one female subject (J. G.), a sharp drop in concentration of FFA occurred three and five hours after the fat meal (Table I). The subject receiving no protein with his breakfast (B. B.) had as marked a drop as the others. All uniformly reported restitution of energy and sense of well-being following the refeeding, with disappearance of weakness and fatigue which had become prominent during the starvation period. In one subject the fat breakfast was repeated one week following the termination of starvation (A. M.). The FFA response was intermediate between the control response and the post-starvation response.

Alimentary rise in triglyceride concentration was slightly lower following the starvation period than in the control period of five of the six subjects.

Response to oral glucose. Table II shows the slight fall in all lipid components and a marked fall in FFA when glucose was given after an overnight fast (control). After three days of starvation the high concentrations of FFA achieved by the fasting fell abruptly following the ingestion of glucose, reaching normal concentrations two and three hours after the glucose. Slight decrease in the concentration of all serum lipid frac-

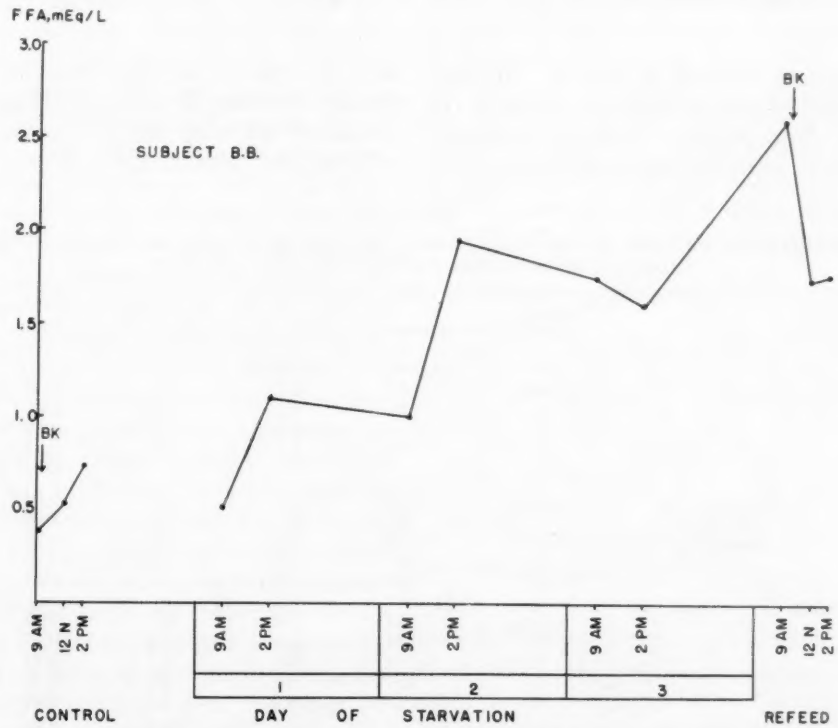

FIG. 1. SUBJECT B. B., PLASMA FREE FATTY ACIDS BEFORE AND AFTER A CONTROL FAT MEAL, DURING A THREE DAY PERIOD OF STARVATION, AND AFTER FEEDING A FAT MEAL AT THE CONCLUSION OF THE STARVATION PERIOD. FFA = free fatty acids; BK = breakfast containing 60 g of fat but no carbohydrate.

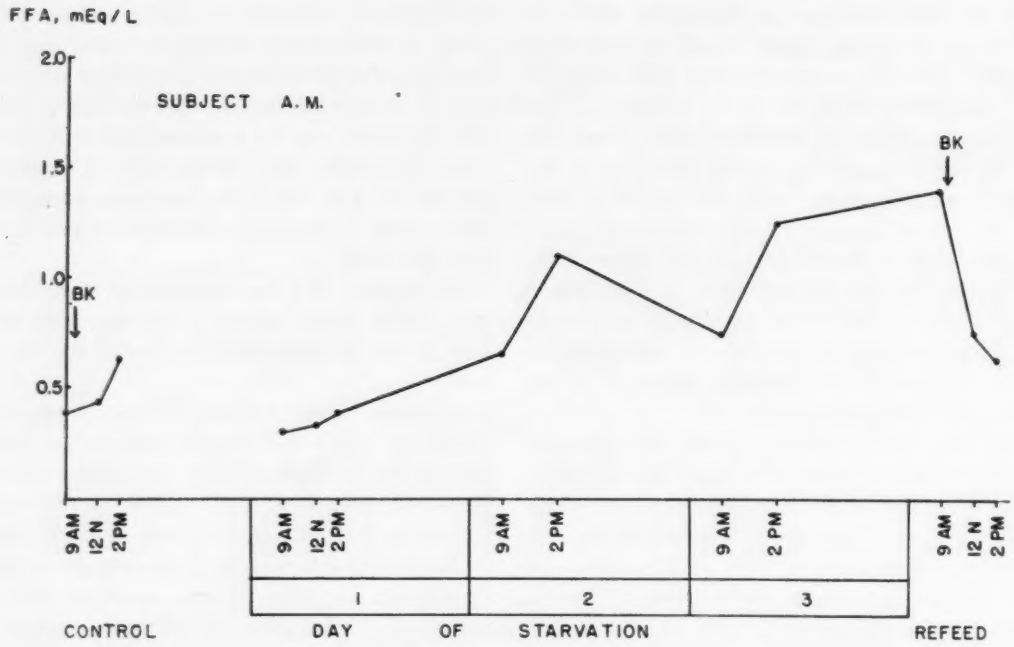

FIG. 2. SUBJECT A. M., PLASMA FREE FATTY ACIDS BEFORE AND AFTER A CONTROL FAT MEAL, DURING A THREE DAY PERIOD OF STARVATION, AND AFTER FEEDING A FAT MEAL AT THE CONCLUSION OF THE STARVATION PERIOD.

tions occurred in response to glucose. In contrast to the normal glucose tolerance curves of the control day, both subjects displayed markedly diabetic types of tolerance curves on the refeeding day. Despite the profound response of FFA to glucose refeeding, the subjects did not experience a sense of well-being such as that reported by the subjects refed with the fat meal.

TABLE II

Changes in plasma lipids and blood sugar following 100 g oral glucose given after an overnight fast (control), and terminating a period of three days' starvation

Subject Sex		Time after glucose	Cholesterol	Lip. P	Triglyceride fatty acids	FFA	Blood sugar
		hrs	*mg/100 ml*	*mg/100 ml*	*mEq/L*	*mEq/L*	*mg/100 ml*
W. P. M	Control	Fasting	161	6.7	1.7	0.34	87
		1	156	6.5	1.9	0.21	106
		2	158	6.6	1.7	0.18	83
		3	150	6.1	1.4	0.18	83
		5				0.63	93
	Refeeding on 4th day of starvation	Fasting	180	8.0	3.7	0.94	66
		1	165	7.1	2.8	0.59	155
		2	165	7.2	3.2	0.34	157
		3	148	7.2	1.0	0.25	109
		5	169	7.5	1.7	0.64	59
D. H. M	Control	Fasting	138	6.6	0.9	0.41	82
		1	129	6.3	1.2	0.17	121
		2	135	6.4	0.5	0.11	79
		3	129	5.5	0.6	0.11	74
		5	132	6.4	1.2	0.70	62
	Refeeding on 4th day of starvation	Fasting	177	7.9	10.7	2.91	52
		1	155	7.1	8.2	1.20	186
		2	156	7.0	7.1	0.49	212
		3	154	6.9	6.9	0.43	216
		5	161	6.8	6.9	0.99	110

DISCUSSION

The abrupt fall in concentration of plasma FFA when a two to seven day fast is terminated by feeding a fat meal is compatible with the hypothesis that fat metabolism becomes more efficient during a period of starvation. This drop is in sharp contrast to the lack of response or even rise in FFA concentration when a fat meal follows a fast of only a few hours or overnight. Evidence currently available supports the theory that a rise in plasma FFA concentration represents mobilization of fat from adipose tissue in response to a caloric deficit (3). Conversely, a decrease in FFA concentration signals caloric satisfaction from exogenous sources with concomitant cessation of delivery of FFA to plasma from adipose tissue. Prior to the present study, only maneuvers which either directly or indirectly increase carbohydrate combustion have been found to "cut off" the release of FFA (3). The failure of a fat meal to produce a fall in plasma FFA concentration under normal circumstances may very well mean that fat alone does not, under these circumstances, provide caloric satisfaction. Following the adaptive changes of starvation, a fat meal evidently does provide caloric satisfaction. The failure of one of the two women in the study to make this adaptation might stem from her sex, her tendency to obesity, or from the brevity of her period of starvation (two days). The decreased alimentary lipemia of all but one subject might be another manifestation of increased fat metabolism, although delayed gastric emptying and slow intestinal absorption cannot be ruled out.

The possible mechanisms whereby starvation increases the efficacy of fat as fuel are worthy of some consideration. Since a greater portion of administered fat is oxidized in briefly starved than in carbohydrate-fed animals (13), it stands to reason that after prolonged starvation, administered fat would be even more completely oxidized with a resultant increase in metabolic rate. If the FFA releasing mechanism responds in some way to energy balance rather than to some specific aspect of carbohydrate metabolism, the effect of refeeding a fat meal might cut off the mobilization of FFA from adipose tissue.

As an alternative explanation for the fall in FFA concentration, the feeding of fat might, by stimulating fat combustion, actually increase the removal rate of FFA from plasma without necessarily influencing the release of FFA from adipose tissue. Although changes in FFA concentration are believed to be entirely due to changes in their rate of release from adipose tissue, Bragdon and Gordon (13) have shown that nutritional status affects the removal rate of FFA and chylomicrons differently in different organs. Fat feeding after starvation might, by greatly stimulating fat combustion, have a sufficiently profound effect on the removal rate by some organ or organs to cause an increase in the overall disappearance rate of FFA.

Inherent to both of these possibilities and consistent with the striking sense of well-being reported by subjects refed with the fat meal is the assumption that fat fed after a period of fasting is more completely oxidized than fat fed after only an overnight fast. The adaptive changes occurring in response to starvation are only partially understood. The overall changes of starvation may be summarized as a conservation of carbohydrate and protein, and an increased facility in the combustion of fat (14). The rising concentrations of cholesterol and triglycerides (15) and FFA (4) during starvation reflect the increased transport and metabolism of fat. These changes are associated with enzymatic alterations (16, 17), and are probably under hormonal control (18–21). A similar metabolic change occurs in animals adapted to a high fat, low carbohydrate diet, and animals so adapted survive starvation longer and with less ketosis than those adapted to a high carbohydrate diet (22). It would seem reasonable to expect that the converse would also hold true, that a period of starvation would facilitate adaptation to a high fat, low carbohydrate diet. The present study offers evidence that such is the case. Studies of the hormonal changes of one of the subjects in the present paper will be reported separately (21). The beneficial effect of fasting and of carbohydrate restriction on the ketosis and lipemia of diabetics was well known before the insulin era (23, 24). A lesser degree of ketosis on the last of repeated episodes of starvation in humans (25) may indicate a similar increased efficiency of fat combustion. An inverse relationship between carbohydrate and fat metabolism is present

in many physiological and pathological conditions (26).

SUMMARY

When six normal subjects who had fasted overnight were fed a meal containing 60 g of fat but no carbohydrate, the plasma FFA increased slightly but consistently in all subjects within five hours after the meal.

A period of starvation of from two to seven days' duration caused an increase in plasma FFA concentration from an average of 0.38 after an overnight fast to an average of 1.85 mEq per L at the end of the fast. Variable increases in concentration of serum cholesterol and triglyceride were also noted.

A meal identical in composition to the control meal was given as the first meal terminating the starvation. In contrast to the control meal, this meal was followed by an abrupt decrease in the elevated concentrations of plasma FFA for five of the six subjects.

The present study thus reports a marked fall in FFA when a fat meal follows a period of starvation. This is in contrast to the small alterations in FFA following a fat meal after usual alimentation, and is a manifestation of the adaptive changes occurring during starvation and indicative of increased efficiency of fat metabolism.

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SUPPRESSION OF URIC ACID SYNTHESIS IN THE GOUTY HUMAN BY THE USE OF 6-DIAZO-5-OXO-L-NORLEUCINE *

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(Submitted for publication September 23, 1959; accepted October 30, 1959)

A substantial number of patients with gout has been shown to produce excessive amounts of uric acid (1–4). This overproduction has been demonstrated by the excretion of greater than normal amounts of uric acid in the urine, or an excessive incorporation of isotopically labeled glycine into urinary uric acid. The present day treatment of tophaceous gout consists primarily in the administration of uricosuric agents to increase the renal excretion of the uric acid formed, with use of colchicine, ACTH gel, or phenylbutazone for management of the acute attacks. Control of the production of uric acid has been of necessity confined to limiting the purine and protein content of the diet (5).

A logical approach to the treatment of those patients who overproduce uric acid would be to inhibit the excessive production by pharmacologic agents. In recent years several compounds have been reported to interfere with purine biosynthesis. Two such compounds, azaserine (*o*-diazoacetyl-L-serine) and 6-diazo-5-oxo-L-norleucine (DON), have been shown to suppress the formation of purines in bacteria (6), and Krakoff and Karnofsky have shown that DON inhibits uric acid production in the pigeon (7). Both compounds are structural analogs of glutamine (see Figure 1) and both have been used in the human as potential chemotherapeutic agents against cancer (8, 9). Using partially purified enzyme systems, Levenberg, Melnick and Buchanan (10) demonstrated that these agents do indeed prevent the synthesis of inosinic acid by blocking the utilization of glutamine in the enzymatic conversion of formylglycinamide ribotide to formylglycinamidine ribotide (Figure 2). DON was found to be the more potent inhibitor of the enzyme system, requiring only 0.025 the concentration of azaserine to obtain the same degree of inhibition. Although glutamine is also required in the first step in purine biosynthesis, these agents are less effective inhibitors of this earlier reaction. Zuckerman, Drell and Levin (11) have shown that azaserine reduced the incorporation of glycine-1-C^{14} into urinary uric acid in gouty humans, but did not alter either the serum urate levels or the urinary excretion of uric acid. The purpose of our study was to observe the effect of the administration of DON on uric acid production in gouty patients, as measured by changes in the serum urate levels and urinary excretion of uric acid, and by the incorporation of glycine-1-C^{14} into urinary uric acid.

METHODS

Six male patients with gout and one with urate renal lithiasis and essential hyperuricemia were studied during hospitalization. They were all maintained on a standard 2,600 calorie, 70 g protein diet, essentially free of purines.

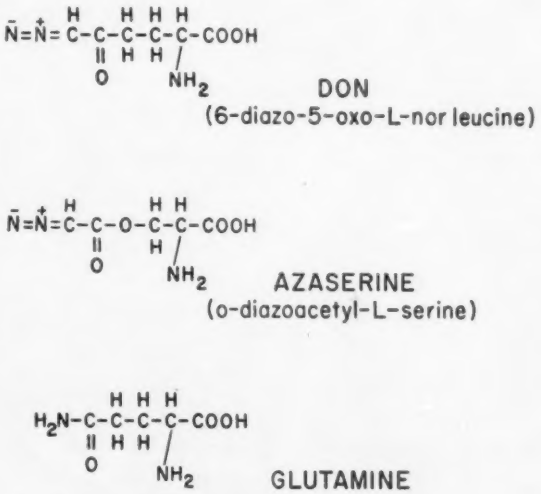

FIG. 1. DON, AZASERINE AND GLUTAMINE, SHOWING STRUCTURAL RELATIONSHIPS.

* A preliminary report of this work has been published in abstract form (J. clin. Invest. 1959, **38**, 1008).

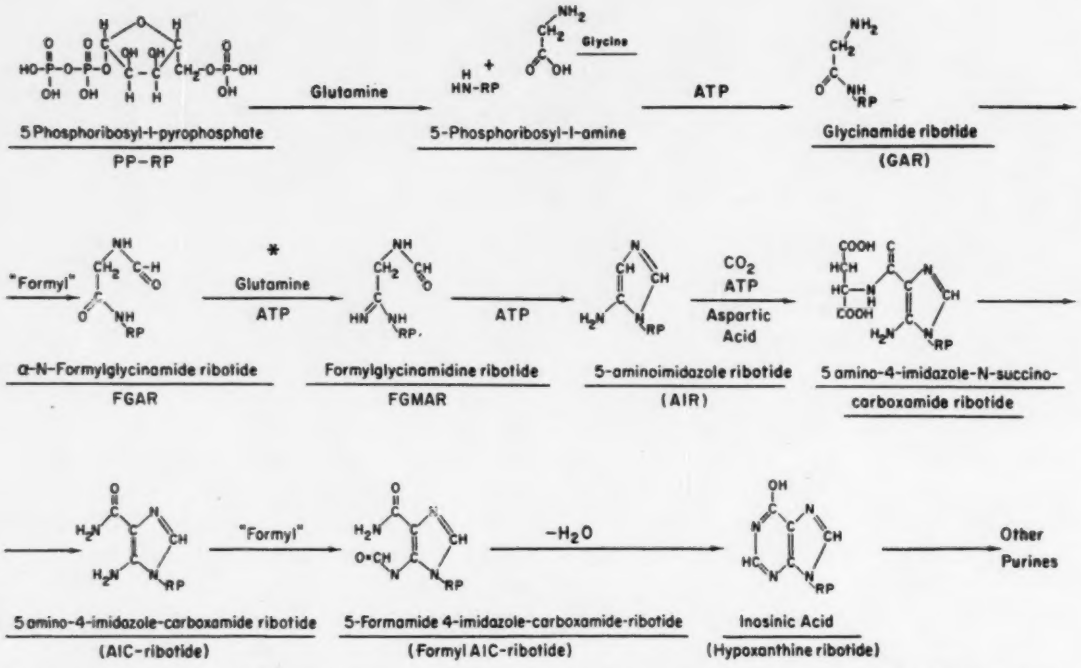

FIG. 2. PATHWAY FOR THE BIOSYNTHESIS OF URIC ACID. Asterisk indicates the enzyme that is inhibited by DON.

At least five days were allowed for equilibration before studies were begun. Daily 24 hour urine collections were preserved with toluene at room temperature. The uric acid determinations of both urine and serum were made by means of an enzymatic spectrophotometric assay (12). Urinary creatinine was determined by the method of Taussky (13). DON, provided by the Cancer Chemotherapy National Service Center was freshly made up in solution at the beginning of each study and stored in the frozen state. It was given by the intramuscular route, either 10 mg daily or in divided doses of 2.5 or 5 mg every six hours. Isotopically labeled compounds were administered on the third day of DON therapy immediately following DON injection. Glycine-1-C^{14} (obtained

TABLE I

Serum urate levels and 24 hour urinary uric acid excretion in patients receiving 6-diazo-5-oxo-L-norleucine (DON)

Patient	Age	Diagnosis	Dose of DON per day	Serum urate		Excretion of uric acid	
				Average of 2 days prior to administration of DON	Lowest while receiving DON	Before DON (average of 3 days)	Lowest while receiving DON
	yrs			*mg/100 ml*	*mg/100 ml*	*mg/24 hr*	*mg/24 hr*
T. B.	39	Essential hyperuricemia and renal urate lithiasis	10 mg	9.5	6.5	1,280	730
S. M.	53	Tophaceous gout	10 mg	9.4	9.9	393	313
R. J.	41	Tophaceous gout	20 mg in divided doses	10.2	9.0	1,140	716
F. J.	34	Tophaceous gout	5 mg	10.9	11.4	1,250	1,110
N. M.	49	Gout	5 mg	10.6	9.7	570	408
J. S.	53	Gout	10 mg	7.9	7.5	598	467
A. H.	48	Tophaceous gout	10 mg	7.5	6.9	494	417

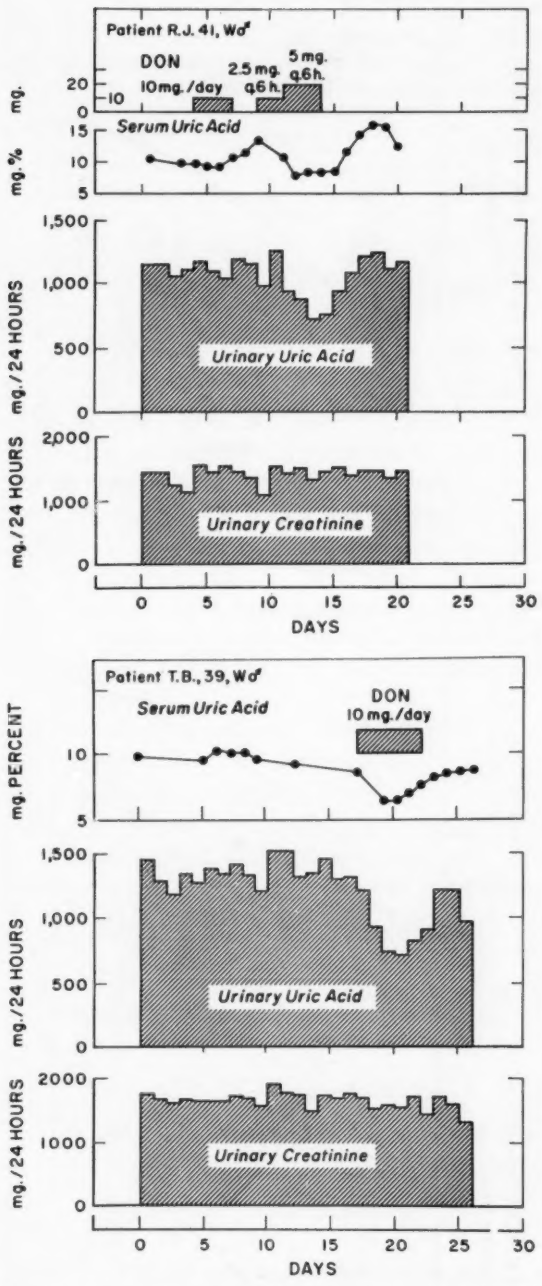

FIG. 3. THE EFFECT OF DON ON SERUM URATE LEVELS AND URINARY URIC ACID EXCRETION.

from Tracerlab Company of Boston), with a specific activity of 1.33 mc per mmole, was given orally in a dose of 5 μc with the breakfast milk. Simultaneously, 36 mg of uric acid N^{15}, containing 28.6 atom per cent excess of N^{15}, was administered intravenously as the lithium salt to measure the urate pool and to allow corrections to be made for altered dynamics of this pool (14).[1] The glycine pool, contributing to hippuric acid, was sampled by administering 450 mg of sodium benzoate intravenously, collecting urine for a two hour period immediately following the injection, and isolating hippuric acid from the specimen. Such sampling was done six hours after the initial administration of glycine-1-C^{14} and during the first two hours of the collection period on the succeeding second, third, fifth and seventh days. A period of at least three months was allowed between control and DON studies to allow for elimination of most of the C^{14} and correction was made for residual C^{14}. Hippuric acid was isolated by extraction with ether and recrystallization from hot water (15). Urinary creatinine, as the double zinc salt (16), was isolated from urine. Uric acid was isolated from aliquots of urine by adsorption on activated charcoal (17), or by preliminary precipitation as a copper salt when urine volumes exceeded 1,500 ml per day (18). Radioactivity was determined using an end-window gas flow counter (Nuclear, Chicago) from planchets plated at infinite thickness with sufficient time for counting to produce an error of not more than 3 per cent. The N^{15} content of uric acid was determined in a Consolidated-Nier mass spectrometer following preliminary digestion (19).

RESULTS

The effect of the administration of DON on the serum urate levels, and on the 24-hour urinary uric acid excretion values in the two patients who demonstrated the greatest response to DON, are shown in Figure 3. The results obtained in all patients are presented in Table I. Even though the data in this table are presented in a manner that would emphasize small differences, i.e., by comparing the average of several days during the pretreatment period with the lowest values obtained during treatment, the differences are not great. In four patients the difference in urinary uric acid excretion was of the order of 20 per cent. Two patients showed a slight increase in serum urate levels during DON therapy. These two patients both had tophaceous gout and the increased miscible urate pool may have prevented the decline of their serum urate levels and urinary uric acid excretion which might otherwise have resulted from a decrease in urate production.

A more sensitive index of purine biosynthesis is provided by the incorporation of glycine-1-C^{14}

[1] We are indebted to Miss Jean Benedict for providing the uric acid-1,3-N^{15}.

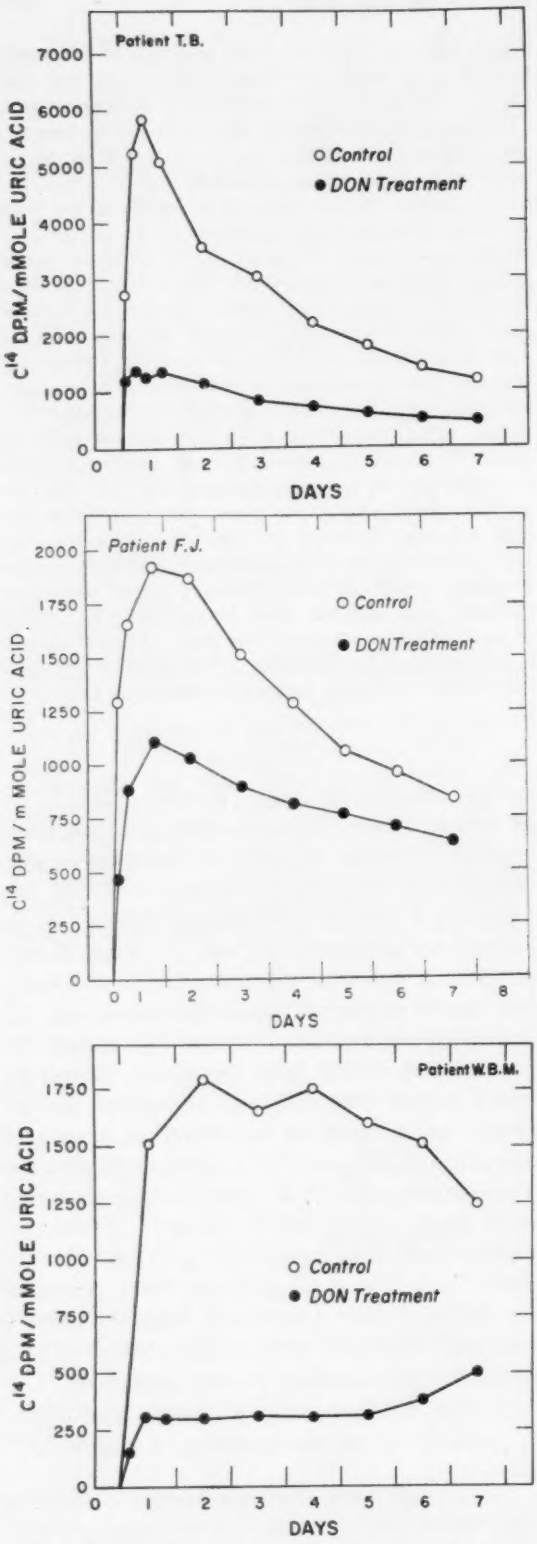

FIG. 4. THE EFFECT OF DON ON THE SPECIFIC ACTIVITY, IN DISINTEGRATIONS PER MINUTE PER MILLIMOLE OF URINARY URIC ACID, RESULTING FROM THE ORAL ADMINISTRATION OF 5 μC OF GLYCINE-1-C^{14}.

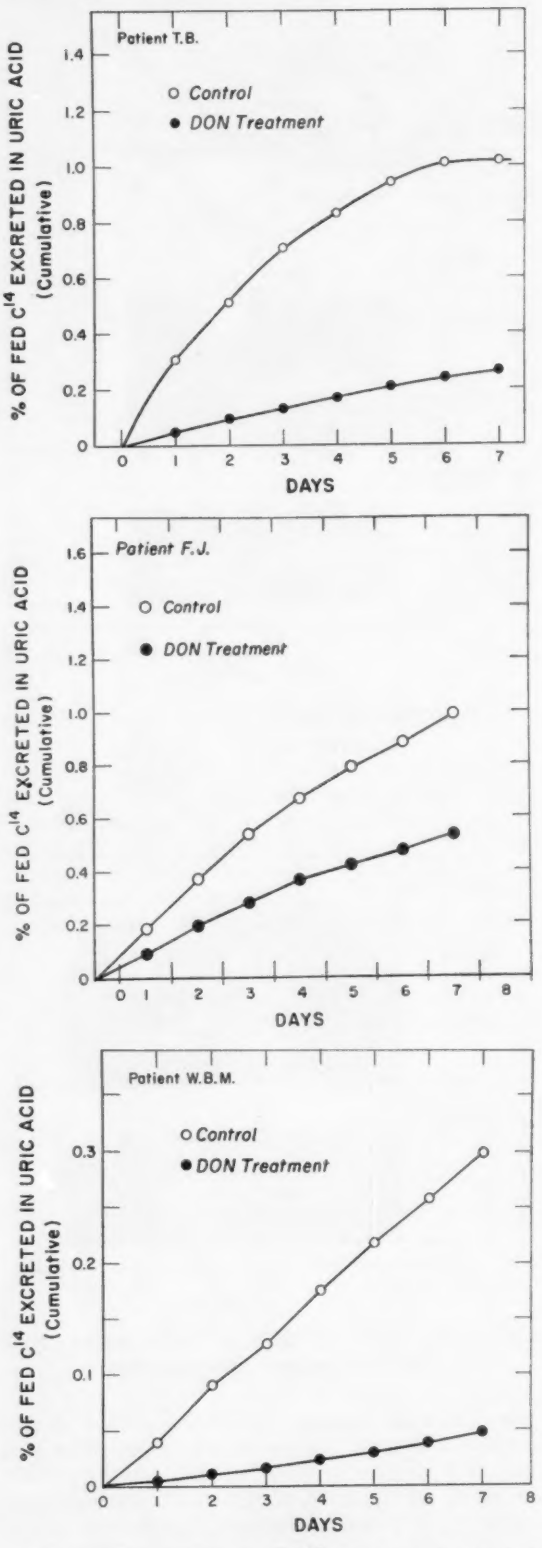

FIG. 5. THE EFFECT OF DON ON THE CUMULATIVE INCORPORATION OF GLYCINE-1-C^{14} INTO URINARY URIC ACID. All values are normalized to the same dose of administered glycine-1-C^{14}.

TABLE II

Suppression of uric acid synthesis from glycine-1-C^{14} by DON

Patient	Per cent recovery of administered isotope in urinary uric acid in 7 days						Effect of DON
	Glycine-1-C^{14} (a)		Uric acid N^{15} (b)		C^{14} corrected for recovery of N^{15} (a/b × 100)		
	Control	DON	Control	DON	Control	DON	Per cent of control incorporation
T. B.	1.11	0.26	77.2	75.2	1.44	0.35	24
F. J.	0.99	0.61	80.6	72.8	1.23	0.84	68
W. M.	0.30	0.05	*	35.7			17

* No N^{15} urate administered.

into urinary uric acid. Figure 4 shows the effect of DON therapy on the specific activity of C^{14} in urinary uric acid; Figure 5 shows the cumulative incorporation of C^{14} into urinary uric acid in three patients. From Table II it is apparent that Patients W. M. and F. J., who failed to show a decline in serum urate during therapy, nevertheless showed a substantially decreased incorporation of glycine-1-C^{14} into uric acid indicative of a depressed purine biosynthesis accompanying DON therapy.

In two of these patients corrections were made for the dynamics of the urate pool, as evidenced by the recovery of injected uric acid N^{15} in urinary uric acid (Table II). By seven days the enrichment of urinary uric acid with N^{15} in each study had declined in a logarithmic manner to values approximating 1.0 per cent of those observed on the first day, except in Patient W. M., in whom the value was 5 per cent. The fraction of N^{15} not recovered in urinary uric acid represents urate removed from the body pool by means other than renal excretion. These would include deposition in tophi, losses via perspiration and gastrointestinal uricolysis. Thus, an estimation can be made of the amount of C^{14} actually delivered to the body's urate pool which did not appear as urinary uric acid. Such a corrected figure represents the best approximation of the extent of purine synthesis *de novo*. In Patient T. B., who received 10 mg of DON per day, the corrected cumulative incorporation was reduced to 24 per cent of the control value. In Patient F. J., 5 mg of DON per day resulted in a reduction to 68 per cent of the control value. The specific activity of the glycine pools sampled by hippuric acid and by creatinine was not significantly altered following DON administration.

TABLE III

Toxic manifestations in patients receiving DON

Patient	Dose of DON	Duration	Toxic manifestations
	mg/day	*days*	
T. B.	10	5	Erosions of oral mucosa on day 5 of therapy
W. M.	10	5	Erosions of oral mucosa on day 5 of therapy
R. J.	10	5	Erosions of oral mucosa on day 3 of higher dose
	20	3	Asymptomatic duodenal ulcer demonstrated by X-ray (Films taken at another hospital 2 months previously showed a deformed duodenum)
F. J.	5	5	None
N. M.	5	9	Erosions of oral mucosa on day 5 of higher dose
	10	6	Asymptomatic duodenal ulcer demonstrated on X-ray
J. S.	10	6	Erosions of oral mucosa on day 6 Negative upper GI X-ray
A. H.	10	6	Shallow erosion of tip of tongue on day 6 Negative upper GI X-ray

The toxic effects consisting of a sore mouth, occasionally with evidence of shallow erosions of the mucous membrane of the under lip, were observed at the end of five days in five patients (Table III). Two patients who received DON for 14 and 7 days, respectively, developed asymptomatic duodenal ulcers which healed promptly on medical therapy once the DON was stopped. One patient, F. J., had no symptoms on a dose of 5 mg per day, although there was an effect on his incorporation of glycine into urinary uric

acid. No depression of the formed elements of the blood, changes in liver function, including the serum glutamic-oxalacetic transaminase level, abnormalities of the urinary sediment, or occult blood in the stools were noted.

DISCUSSION

DON had a marked effect on purine biosynthesis in the human, as shown by a substantial reduction in the incorporation of glycine-1-C^{14} into urinary uric acid in all three patients studied. Such findings are consistent with the interpretation that DON exerts an effect *in vivo* in the human similar to the effect observed in other biological systems. Presumably, such effects result from its action in blocking the enzymatic conversion of formylglycinamide ribotide to formylglycinamidine ribotide. That DON has an effect on other glutamine-requiring reactions (20) leaves open the possibility that other, more specific inhibitors of purine biosynthesis may be found having fewer undesirable side effects.

DON administration failed to affect significantly the specific activity of the glycine pool sampled by hippuric acid and by creatinine. This is evidence that absorption of glycine-1-C^{14} from the intestine was unaffected by DON, and further supports the view that the action of DON, in the dosage used in this study, is primarily a suppression of purine synthesis. The failure of balance studies to demonstrate a marked reduction in serum urate levels or urinary uric acid excretion in five of the seven patients studied has several possible explanations. With the short periods of drug administration and the presence of a large miscible urate pool, a balance study may fail to reflect brief changes in uric acid synthesis because of the dampening effect of such a pool. The failure to obtain any decrease in serum urate levels in Patients R. J. and W. N., despite a substantial suppression of purine biosynthesis, as shown by reduced glycine incorporation, might be explained by the fact that these two patients had the largest tophaceous deposits of all the patients studied. Another possible explanation may be related to the relatively short half-life of DON in the blood (approximately three to four hours following its intramuscular administration) (9). Thus, maximal effects of DON were obtained at a time when glycine-1-C^{14} in the body had its highest specific activity. A compensatory overproduction of uric acid during the remainder of the day may have masked such a suppression at a gross level.

Upon discontinuing DON therapy, the serum urate showed a rebound, similar to that shown in Figure 3 (Patient R. J.), to values higher than those of the control period in five of the seven patients studied. Such transient elevations may well be related to the operation of a homeostatic control of purine synthesis (21, 22) similar to that described for pyrimidine synthesis in bacteria (23). A derangement of such homeostatic control can be produced by the drug 2-ethylamino-1,3,4-thiadiazole (24, 25). A constitutional derangement in such a controlling mechanism has been proposed to account for the increased uric acid synthesis seen in some cases of gout. A suppression of purine biosynthesis has also been produced by administering 4-amino-5-imidazolecarboxamide (21). This substance, however, is itself converted to uric acid and so is of no value in treatment of the excessive production of uric acid seen in gout.

A considerable variability in responsiveness of different patients to a given dose of DON makes evaluation of its therapeutic index difficult. DON given for five days at a dose of 5 mg per day to Patient F. J. had no untoward side effects. The same dose continued for nine days in the second patient, N. M., again produced no untoward side effects, but when the dose was increased to 10 mg per day, an asymptomatic duodenal ulcer developed after five days. Although DON administered in higher doses as chemotherapy for cancer results in a significant incidence of leukopenia, thrombocytopenia, nausea and vomiting (9), no such toxic effects were observed during this study. The relatively high incidence of oral toxicity and peptic ulceration, even at relatively low doses, makes the use of DON imprudent in managing patients who overproduce uric acid. This approach, however, of reducing excessive uric acid production by means of inhibitors of purine biosynthesis appears to be possible, and warrants a further search for a more specific inhibitor with a more favorable therapeutic index.

SUMMARY

DON (6-diazo-5-oxo-L-norleucine) has been administered to six gouty patients and to one pation with renal urate lithiasis and essential hyperuricemia. *1*) Studies performed on three patients demonstrated that DON suppressed the incorporation of glycine-1-C^{14} into urinary uric acid. *2*) Balance studies demonstrated that in two patients there was a significant decrease in serum urate levels and 24-hour urinary uric acid excretion following the administration of DON. *3*) From the above studies, it is concluded that DON suppresses uric acid biosynthesis, but such suppression is not always reflected in a short-term balance study, especially in the presence of an expanded urate pool. *4*) The use of pharmacologic agents to suppress synthesis in those gouty patients who overproduce uric acid represents a new and rational approach to their therapy. The high incidence of side effects following DON administration prevents the practical use of this particular drug.

ACKNOWLEDGMENT

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ANNOUNCEMENT OF MEETINGS

The Fifty-Second Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Sunday afternoon and evening, May 1, 1960, in the Chalfonte-Haddon Hall (jointly with the AFCR); and on Monday, May 2, at 9:00 A.M., at the Casino Theater on the Steel Pier.

The Seventeenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., on Sunday, May 1, 1960, at 9:00 A.M., at the Casino Theater on the Steel Pier. On Sunday afternoon and evening, May 1, 1960, a joint sectional meeting with The American Society for Clinical Investigation will be held in rooms in the Chalfonte-Haddon Hall.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold its Seventy-Third Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 3, 1960, at 9:30 A.M., and in the Vernon Room, Chalfonte-Haddon Hall, on Wednesday, May 4, 1960, at 9:30 A.M.

SERUM LIPID AND LIPOPROTEIN ALTERATIONS IN NEPHROSIS

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When nephrosis was recognized as a clinical entity, an increased concentration of serum cholesterol was observed to be a consistent feature of the syndrome (1). Later it was found that phospholipid and triglyceride also are increased in nephrosis (2–5).

The increase in serum lipids in nephrosis is usually greatest in the cases with the lowest levels of serum albumin. It has not been established conclusively, however, that the hyperlipidemia is caused by hypoalbuminemia, and irregularities in the relationship between levels of serum lipids and serum albumin have been pointed out repeatedly (2–5).

After it was recognized that lipids exist in the serum in the form of lipoprotein macromolecules, techniques developed for the separation and characterization of proteins were applied to studies of lipoproteins. An abnormal distribution of cholesterol among the serum protein fractions precipitated by alcohol was found by Barr, Russ and Eder (6) in cases of nephrosis. Kunkel and Slater (7) observed an abnormal pattern of serum lipoproteins by zone electrophoresis—in part a result of increased quantities of unesterified fatty acid bound to the lipoproteins (8)—in nephrosis. Gofman, Rubin, McGinley and Jones (9) noted in nephrotic subjects that all classes of lipoproteins studied by them in the analytical ultracentrifuge (S_f 0 to 400) were increased.

In the present study lipoprotein fractions of sera from patients with nephrosis were isolated by the technique of ultracentrifugation at various densities and analyzed for lipids and protein. Levels of individual serum lipids are correlated with lipoprotein pattern, and also with serum lactescence and serum albumin concentration. It is emphasized that lipoprotein patterns of different sera may differ greatly even when serum cholesterol levels are the same.

METHODS

Subjects. Forty-four hospitalized patients with nephrosis or the nephrotic syndrome were studied. In a majority of cases the disease was considered to be idiopathic. There was one case of systemic lupus erythematosus (N.G.) and one of primary amyloidosis with renal involvement (M.K.). Symptoms of nephrosis in different cases ranged from severe to mild. Clinical features of many of the cases have been described in detail elsewhere (10). Most of the patients were ambulatory and were taking food satisfactorily. Sera obtained during periods of severe complications, experimental procedures, or therapy (other than sodium restriction and symptomatic care) were excluded. Likewise, sera obtained soon after admission to the hospital or soon after a change in clinical condition of the patient were excluded if the results were shown by repeated subsequent analyses to be aberrant. Results of analyses of 2 or 3 similar sera from the same patient are included in the tables in some instances but mean values for these duplicate sera are used in the charts and calculations. In the case of almost one-half of the patients, 2 or occasionally 3 dissimilar sera obtained in different stages of the disease, sometimes before and after complete or partial remissions, are included and counted individually. In a majority of cases the serum albumin and cholesterol levels which are recorded were shown by serial determinations to be approximately steady state values. Repeated determinations of the other lipids also were made in more than one-half of the cases.

Blood samples and analytical procedures. Blood specimens were taken by venipuncture about 7:30 a.m. after an overnight fast. Analyses of whole sera for lipids were made by methods previously employed in this laboratory (11, 12), using ethanol-acetone for extraction of lipids in the analyses done prior to 1956 and chloroform-methanol (13) in the more recent analyses. Total lipid was determined by the method of Bragdon (14), free and total cholesterol by the Schoenheimer-Sperry method (15), and lipid phosphorus by a modification of the method of Stewart and Hendry (16). Phospholipid was calculated as lipid phosphorus times 25. Triglyceride was calculated by difference (14), with a correction for interference by nonlipid substances in cases of extracts made with ethanol-acetone (13). Serum albumin was determined by a modification of the dye interaction

TABLE I

Serum lipoprotein fractions studied

Density	Lipoprotein fraction	Electrophoretic mobility (starch)
<1.019	Very low density $S_f > 10$	α_2–β_1 Globulin
<1.006	$S_f > 17$	
1.006–1.019	S_f 10–17	
1.019–1.063	Low density S_f 0–10	β_1 Globulin
1.063–1.21	High density	α_1 Globulin
>1.21	Residual serum components including proteins, unesterified fatty acid (bound to albumin), and a phosphorus-containing substance soluble in ethanol-acetone.	

method of Rutstein, Ingenito and Reynolds (17), and checked in some cases by electrophoresis.

Lipoprotein fractions of the sera were isolated by preparative ultracentrifugation at various densities followed by separation of the supernatant from the infranatant material by slicing of the tubes (18). For this purpose 5 ml aliquots of sera were adjusted successively to densities of 1.019, 1.063, and 1.21 by addition of solutions of sodium chloride and potassium bromide and centrifuged at 105,000 × G at 15° C in the no. 40 rotor of a Model L Spinco ultracentrifuge for 18, 24, and 48 hours successively (11, 12). In some cases an additional (initial) fractionation at density of 1.006 (D 1.006) was made, after dilution of the sera with 0.15 M sodium chloride solution. The equivalent fractions obtained by other methods are shown in Table I. Protein was determined directly or after extraction of the fractions with ethanol-acetone by a biuret method (19) or by the method of Lowry, Rosenbrough, Farr and Randall (20). Lipids in the fractions were determined as described above for whole serum. The recoveries of cholesterol and phospholipid in the lipoprotein fractions averaged about 95 per cent of the quantities in the original sera. Triglyceride values for the D 1.019–1.063 and D 1.063–1.21 fractions are not shown but may be calculated approximately by assuming, respectively, that 25 and 17 per cent of total cholesterol was free.

The lipids and lipoprotein fractions in sera of normal young adults have been studied previously using the methods employed in this study (11–13). These normal values are utilized as a standard of reference for the present results. Ages of the patients varied but the abnormalities in lipid values were so great that normal variations with age were relatively unimportant.

RESULTS

Serum lipids

The sera are divided into two categories on the basis of total cholesterol (TC) concentration: Category A with definitely increased lipids (TC > 350 mg per 100 ml), and Category B with normal or only slightly increased lipids (TC < 300 mg per 100 ml). Results of analyses of sera of Category A are shown in Table II. Except for minor rearrangements in order to place together similar (duplicate) sera from the same patients, the sera are listed in order of decreasing concentrations of triglyceride. In listing the sera in this manner they are also arranged approximately in order of decreasing lactescence. For convenience of discussion, the sera of Category A are subdivided into four successive groups (Groups 1, 2, 3 and 4). The description of the lipid and lipoprotein abnormalities which follows is based principally on observations on these sera.

The results of analyses of sera of Category B—those with normal or slightly increased lipids—are shown in Table III. Some of these sera were obtained from patients with proteinuria of moderate degree (Group 5) and others were taken during periods of partial or complete remission of the disease with little or no proteinuria (Group 6). These slightly abnormal sera are included to complete the picture of the lipid alterations in nephrosis and to extend the range over which values of serum lipids and serum albumin may be correlated. The division of all the sera of the study into categories and groups is shown graphically in Figure 1, and mean results of analyses of the sera of the various groups are presented as multiples of normal values (11, 12) in Figure 2.

Total cholesterol concentration as already noted was above 350 mg per 100 ml in all sera of Category A. Mean TC was greatest in Group 1 and progressively less in the other groups. The range of values in all groups of Category A overlapped, however, and mean values for Groups 2, 3 and 4 were not greatly different. Free cholesterol (FC) in all sera was increased at least in proportion to the increase in TC, and in the lactescent sera a greater than normal fraction of total cholesterol was free. Phospholipid (PL) was increased whenever TC was increased but to a lesser degree; the ratios of TC/PL were greater than normal in all sera with increased lipids and were greatest in the sera with the greatest concentrations of TC (Figure 3). Values of TC/PL were correlated on average with the degree of lactescence of the sera but the relationship was in-

TABLE II

Data on sera of Category A, with total cholesterol greater than 350 mg per 100 ml

Serum no.	Patient	Age and sex	Date	TC*	PL*	TG*	TL*	FC/TC	TC/PL	TC/TG	Albumin
				mg/100 ml							*g/100 ml*
Approx. normal values for young adults				179	226	100	580	0.25	0.79	1.8	4.0
				Group 1 (very lactescent)							
1	J. H.	2 M	11/15/54	677	620	3,820	5,439	0.30	1.09	0.2	0.6
2	D. B.	2 F	10/12/54	1,058	1,064	3,220	5,817	0.34	0.99	0.3	0.4
3	S. S.	33 M	2/15/55	1,522	942	3,210	6,409	0.29	1.63	0.5	0.8
4	L. G.	29 M	2/25/55	1,234	785	2,440	5,063	0.28	1.57	0.5	0.6
5	W. T.	10 M	7/ 5/57	878	671	2,103	4,070	0.30	1.31	0.4	0.5
6	W. T.	10 M	6/12/57	774	650	2,074	3,872	0.29	1.19	0.5	0.5
7	C. D.	7 F	2/15/56	1,174	848	2,377	4,966	0.29	1.39	0.5	0.4
8	C. P.	6 M	7/29/55	1,293	968	2,359	5,253	0.28	1.34	0.5	0.9
Mean				1,112	841	2,788	5,274	0.30	1.32	0.4	0.60
				Group 2 (lactescent)							
9	R. B.	6 F	4/ 1/58	698	641	1,204	2,885	0.28	1.09	0.6	0.7
10	R. B.	6 F	4/ 7/58	659	559	1,151	2,687	0.29	1.18	0.6	0.8
11	J. S.	4 M	7/21/58	693	552	1,111	2,686	0.30	1.25	0.7	0.6
12	J. S.	4 M	7/24/58	736	552	950	2,595	0.29	1.33	0.7	0.6
13	F. G.	45 M	2/10/59	659	633	1,059	2,653	0.33	1.04	0.6	1.0
14	N. G.	45 F	3/11/58	405	410	739	1,746	0.30	0.99	0.6	2.2
15	N. G.	45 F	2/24/58	402	396	581	1,570	0.30	1.01	0.7	2.3
16	C. T.	36 M	7/22/57	639	456	825	2,233	0.28	1.40	0.8	0.8
17	C. T.	36 M	7/10/57	628	469	700	2,096	0.30	1.34	0.9	0.9
18	C. T.	36 M	7/20/57	620	458	694	2,075	0.28	1.35	0.9	0.9
19	G. W.	13 M	7/17/58	868	619	627	2,545	0.27	1.40	1.4	0.3
Mean				659	545	882	2,403	0.29	1.21	0.8	0.97
				Group 3 (slightly lactescent)							
20	W. G.	61 M	2/15/56	755	523	583	2,365	0.29	1.44	1.3	0.8
21	O. H.	41 M	2/ 6/55	588	497	570	1,955	0.25	1.18	1.0	1.1
22	K. R.	14 M	10/ 3/57	703	537	551	2,140	0.27	1.31	1.3	0.7
23	J. J. S.	9 M	9/ 9/58	427	379	485	1,494	0.30	1.12	0.9	1.1
24	E. W.	50 M	2/25/55	857	691	480	2,443	0.29	1.24	1.8	0.9
25	R. S.	5 M	3/12/57	710	602	432	2,106	0.25	1.18	1.7	0.8
26	R. S.	5 M	2/28/55	682	592	390	2,021	0.23	1.21	1.6	0.6
27	D. Z.	19 M	7/10/56	369	353	427	1,330	0.28	1.04	0.9	1.6
28	R. B.	6 F	5/12/58	502	462	414	1,630	0.26	1.09	1.2	1.0
29	M. K.	54 M	2/15/56	556	475	409	1,722	0.28	1.17	1.4	1.6
30	F. F.	30 F	11/25/54	471	419	400	1,424	0.27	1.12	1.2	1.0
31	M. H.	27 F	9/16/58	430	404	386	1,419	0.32	1.06	1.1	1.3
32	W. T.	10 M	3/ 1/58	409	347	370	1,335	0.25	1.18	1.1	1.5
33	M. D.	6 M	3/13/56	566	481	348	1,616	0.26	1.18	1.6	2.0
34	A. R.	7 M	1/ 7/58	590	477	331	1,695	0.26	1.24	1.8	0.8
35	O. C.	68 M	2/24/58	378	377	324	1,269	0.26	1.00	1.2	1.2
36	B. G.	16 F	8/ 3/54	354	354	320	1,208	0.25	1.00	1.1	1.2
37	K. R.	14 M	9/19/57	1,053	700	294	2,591	0.24	1.50	3.6	0.8
38	F. N.	65 M	1/27/59	625	479	266	1,508	0.27	1.30	2.4	1.0
Mean				574	475	409	1,734	0.27	1.19	1.5	1.13
				Group 4 (clear)							
39	J. W.	3 M	2/24/54	364	320	218	1,083	0.27	1.14	1.7	2.8
40	W. T.	11 M	2/26/58	459	366	205	1,270	0.23	1.26	2.2	1.2
41	J. M.	4 F	3/21/57	590	464	199	1,554	0.25	1.27	3.0	1.9
42	S. S.	35 M	3/25/57	379	399	197	1,171	0.24	0.95	1.9	2.5
43	S. S.	34 M	2/15/56	604	457	189	1,546	0.28	1.32	3.2	2.3
44	L. N.	21 F	6/27/55	464	368	189	1,245	0.28	1.25	2.4	1.0
45	E. G.	44 M	2/10/54	438	352	186	1,202	0.24	1.24	2.4	1.5
46	D. P.	3 M	4/ 5/57	703	514	174	1,747	0.24	1.37	4.0	0.9
47	F. F.	32 F	5/23/56	468	384	172	1,269	0.23	1.22	2.7	1.7
48	E. T.	9 M	2/ 4/54	469	394	170	1,285	0.21	1.19	2.8	1.2
49	E. T.	9 M	1/25/54	518	391	145	1,325	0.23	1.32	3.6	1.6
50	M. E. H.	5 F	4/ 9/57	678	499	169	1,683	0.27	1.36	4.0	1.2
51	M. C.	14 F	8/22/56	676	538	158	1,703	0.28	1.25	4.3	0.6
52	S. J.	15 M	6/26/58	373	325	140	1,030	0.24	1.15	2.7	2.8
53	A. R.	4 M	5/18/55	533	493	160	1,476	0.20	1.08	3.3	1.6
54	A. R.	4 M	4/20/55	586	479	120	1,500	0.21	1.22	4.0	2.0
55	C. T.	36 M	10/23/57	357	310	141	992	0.24	1.15	2.5	2.7
56	H. B.	50 M	10/29/58	432	383	132	1,165	0.26	1.13	3.3	1.3
57	O. H.	41 M	7/ 3/56	351	359	75	971	0.22	0.98	4.7	2.3
58	L. N.	21 F	8/16/55	434	378	36	1,072	0.24	1.15	12.5	1.2
Mean				490	404	158	1,302	0.25	1.20	3.7	1.69

* TC = total cholesterol; PL = phospholipid; TG = triglyceride; TL = total lipid.

TABLE III

Data on sera of Category B, with total cholesterol less than 300 mg per 100 ml *

Serum no.	Patient	Age and sex	Date	TC	PL	TG	TL	FC/TC	TC/PL	TC/TG	Albumin
				mg/100 ml							*g/100 ml*
				Group 5							
59	R. T.	34 M	5/19/58	266	330	495	1,220	0.29	0.81	0.5	2.8
60	M. F.	17 F	5/19/58	269	249	106	761	0.25	1.1	2.5	2.4
61	M. F.	17 F	5/27/58	193	225	161	676	0.26	0.86	1.2	2.3
62	W. T.	10 M	10/16/57	189	219	141	645	0.25	0.86	1.3	3.1
63	M. C.	14 F	3/ 8/57	285	268	142	836	0.27	1.1	2.0	2.4
64	M. C.	14 F	4/30/57	263	260	110	766	0.26	1.0	2.4	2.5
65	C. R.	30 M	1/ 9/57	232	222	104	672	0.28	1.0	2.2	2.6
66	E. T.	9 M	5/ 8/54	209	214	107	668	0.27	0.98	2.0	2.9
67	C. D.	7 F	7/11/56	286	288	95	817	0.24	0.99	3.0	2.2
68	R. Q.	3 M	12/18/56	292	282	70	795	0.24	1.0	4.2	3.2
Mean				247	257	159	792	0.26	0.97	2.2	2.71
				Group 6							
69	R. K.	7 M	5/23/56	183	247	267	793	0.23	0.74	0.7	3.5
70	R. K.	7 M	8/15/56	178	220	204	692	0.26	0.81	0.9	3.3
71	D. B.	3 F	10/12/55	241	231	192	785	0.26	0.92	1.2	3.4
72	L. G.	30 M	7/11/56	218	220	190	740	0.25	0.99	1.1	3.9
73	C. J.	2 F	2/ 2/55	174	214	160	638	0.24	0.81	1.1	4.1
74	K. R.	14 M	12/ 9/57	184	223	144	644	0.25	0.83	1.3	3.8
75	E. W.	51 M	7/25/56	229	213	105	669	0.21	1.1	2.2	4.0
76	E. K.	2 F	6/20/56	178	211	92	571	0.25	0.84	1.9	3.8
77	J. W.	30 M	7/11/55	262	254	86	734	0.26	1.0	3.0	3.9
78	R. S.	4 M	6/ 6/56	188	219	83	584	0.27	0.86	2.3	3.5
79	R. Q.	2 M	9/ 7/55	193	194	75	568	0.19	1.0	2.6	4.0
80	A. R.	5 M	2/26/56	172	190	72	522	0.21	0.91	2.4	3.8
81	E. G.	46 M	5/29/56	189	250	76	607	0.27	0.76	2.5	3.3
82	E. G.	46 M	7/11/56	222	252	57	649	0.22	0.88	3.9	3.2
Mean				202	221	125	652	0.24	0.91	1.9	3.74

* For abbreviations, see footnote to Table II.

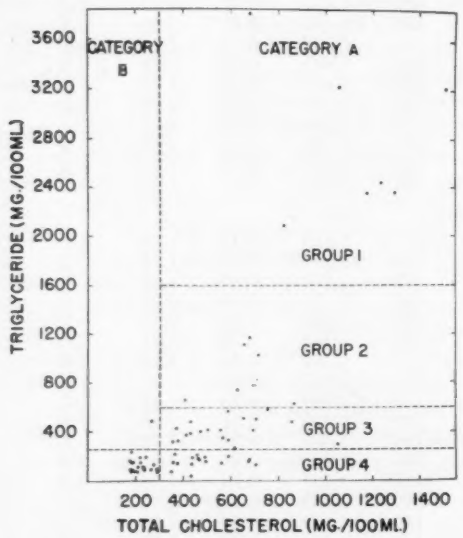

FIG. 1. RELATIONSHIP BETWEEN SERUM TRIGLYCERIDE AND SERUM TOTAL CHOLESTEROL. The various categories and groups of sera are indicated.

consistent among the individual sera. Lactescence was related to the ratio of total lipid (TL) to PL, as noted by Ahrens and Kunkel (21), and less regularly to TL/TC. Lactescence also was related to the level of triglyceride, which explains in part the relationship of lactescence to TL/PL and to TL/TC.

While TC and PL were clearly in excess of normal in all sera of Groups 1 through 4 with some similar concentrations in each group, triglyceride (TG) ranged from very high levels in Group 1 to approximately normal levels in Group 4, with distinctly different ranges of values in the different groups. Ratios of TC/TG varied from a fraction of normal in Group 1 to several times normal in Group 4, and, among sera with TC concentrations in the vicinity of 700, concentrations of TG ranged all the way from 120 to 3,820 mg per 100 ml. All of the lipids increased together on average but the relation-

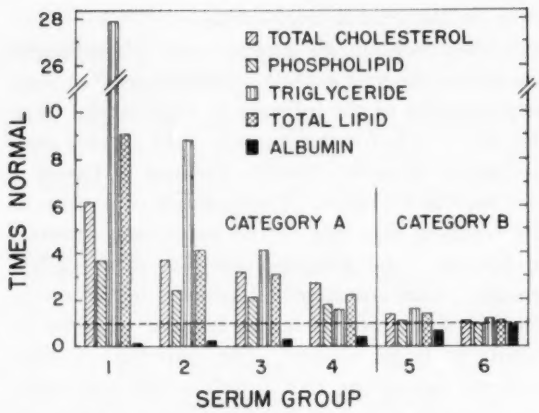

FIG. 2. MEAN CONCENTRATIONS OF LIPIDS AND ALBUMIN IN SERA OF THE VARIOUS GROUPS IN RELATION TO NORMAL CONCENTRATIONS. The normal values are shown in Table II.

ship between TC and TG was very irregular as may be seen in Figure 1. Thus there were qualitative as well as quantitative differences in the lipids in the different sera. These qualitative differences were related to differences in lipoprotein pattern which are discussed below.

Relationship between serum lipids and serum albumin

The concentrations of the various classes of lipids in the sera are shown in relation to the

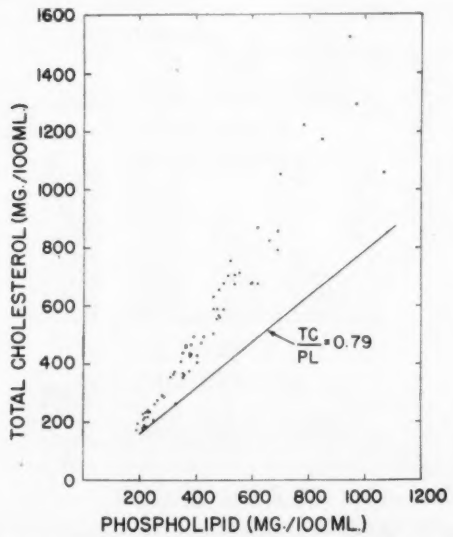

FIG. 3. RELATIONSHIP BETWEEN SERUM TOTAL CHOLESTEROL AND SERUM PHOSPHOLIPID. The normal ratio of TC/PL is shown.

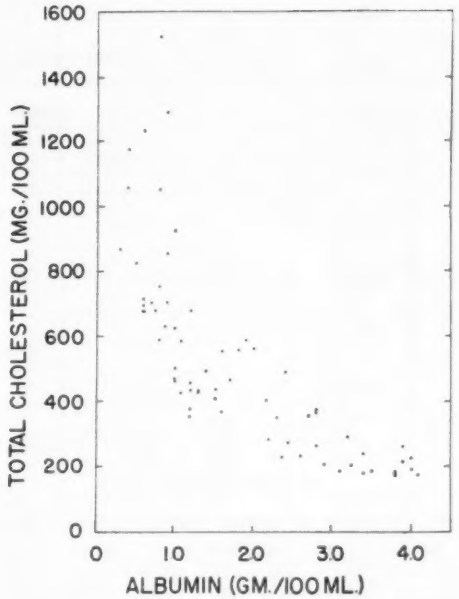

FIG. 4. RELATION OF SERUM TOTAL CHOLESTEROL TO SERUM ALBUMIN.

corresponding concentrations of serum albumin in Figures 4 through 6. It is evident that inverse correlations existed in each case. The relationships were approximately hyperbolic, with the lipids, particularly triglyceride, increasing sharply as albumin decreased below levels of about 1 g per 100 ml. Reciprocals of total cholesterol and of phospholipid concentrations were associated linearly with albumin concentrations with correlation

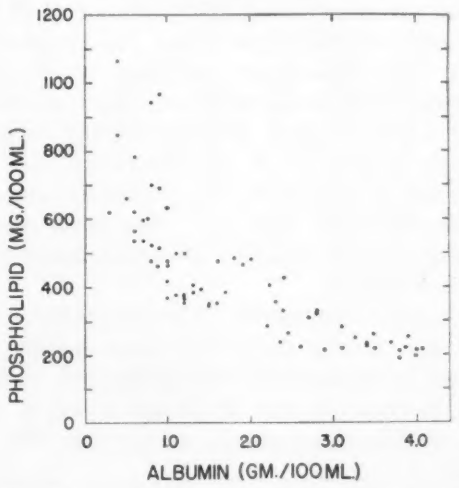

FIG. 5. RELATION OF SERUM PHOSPHOLIPID TO SERUM ALBUMIN.

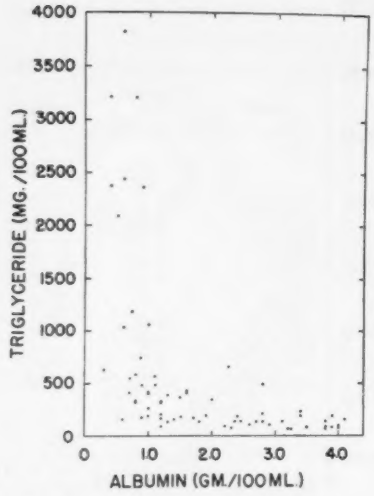

FIG. 6. RELATION OF SERUM TRIGLYCERIDE TO SERUM ALBUMIN.

coefficients (R) of at least + 0.90. The relationship between triglyceride and albumin was less regular, and more suggestive of the importance of a critical concentration of albumin. The relationship between total lipid and albumin resembled in form and regularity the relationship between TC or PL and albumin more closely than it resembled that between TG and albumin.

Serum lipoproteins

The lipoprotein fraction which was isolated in the top portion of the tubes by centrifugation of the sera at a density of 1.019 included all of the lactescent material of the sera. The lipoproteins which were subsequently separated at D 1.063 were clear and often yellow, and those separated at D 1.21 were clear and only slightly colored. Results of analyses of the lipoprotein fractions from sera of each of the four groups of Category A are shown in Table IV. The mean results are expressed as multiples of normal values (11, 12) in Figure 7.

D < 1.019 lipoproteins. Concentrations of the very low density lipoproteins were greatly increased in the lactescent sera of Group 1, moderately increased in sera of Groups 2 and 3, and only slightly above normal in the clear sera of Group 4.

This fraction varied from group to group in composition as well as in quantity. It contained most of the excess triglyceride of the sera, but its cholesterol (in all groups) and phospholipid (in all but the first group) were increased at least in proportion to the increase in triglyceride. Ratios of TC/PL for the fraction were greater than normal in all serum groups, greatest in Group 1 and least in Group 4. The quantity of protein in the fraction, like that of the lipids, was greatest in Group 1 and progressively less in the other groups. Concentrations of protein in Groups 2 through 4 are not presented because of some irregularly large values. The patterns of lipoproteins (according to density) within this heterogeneous fraction and the composition of different portions of the fraction were not investigated extensively. Results of a subfractionation are discussed in a later section.

D 1.019–1.063 lipoproteins. In contrast to the trend of the D < 1.019 lipoproteins described above, there was a stepwise increase in quantity of the D 1.019–1.063 fraction from Group 1 to Group 4. This medium-density fraction was near normal concentration in Group 1, irregularly in-

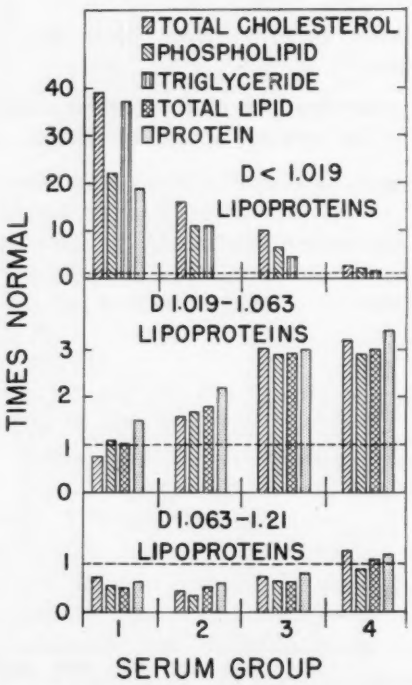

FIG. 7. MEAN CONCENTRATIONS OF LIPIDS AND PROTEIN OF THREE LIPOPROTEIN FRACTIONS IN SERA OF VARIOUS GROUPS IN RELATION TO NORMAL CONCENTRATIONS. The normal values are shown in Table IV.

TABLE IV

Data on lipoprotein fractions of four groups of sera with increased lipids *

Serum no.	Lipoprotein fraction															
	D < 1.019					D 1.019–1.063					D 1.063–1.21					
	TC	PL	TG	FC/TC	TC/PL	TL	TC	PL	P	TC/PL	TL	TC	PL	P	TC/PL	
	mg/100 ml of serum					*mg/100 ml of serum*					*mg/100 ml of serum*					
Approx. normal values	23	28	78	0.37	0.82	265	103	74	70	1.39	190	49	101	170	0.48	
Group 1																
1	609	482	(3,700)†		1.27		52	86	96	0.61		16				
2	907	791	(3,100)		1.15		90	87		1.03		82				
3	1,247	699	(3,100)		1.79		98	99	109	0.99		46	85	121	0.54	
4	1,023	591	(2,300)		1.73		78	81	96	0.96		21	55	120	0.38	
5b‡	624	527	2,485	0.37	1.18	265	63	75	53	0.84	104	19	47	91	0.40	
5a	655	535	2,422	0.35	1.22	265	54	62	95	0.87	69	9	17	56	0.41	
5	778	527	2,021	0.31	1.47	281	77	67	128	1.15	99	17	37	95	0.46	
Mean	894	619	2,901	0.34	1.45	270	77	84	103	0.91	91	36	58	107	0.45	
Group 2																
9	386	361	1,047	0.34	1.07	503	184	131	170	1.40	165	32	51	136	0.63	
11	412	336	1,013	0.34	1.22	651	216	164	219	1.32	53	9	22	31	0.41	
12	406	278	876	0.31	1.46	548	203	142	198	1.43	67	11	22	65	0.50	
13	404	379	903	0.35	1.07	685	212	190	187	1.11	80	12	30	51	0.40	
14	244	222	590	0.35	1.10	309	96	80	134	1.20	119	28	48	146	0.58	
15	237	222	522	0.35	1.07	313	115	87	125	1.32	71	13	32	89	0.41	
16	507	341	799	0.31	1.49	322	103	83	61	1.24	116	24	47	119	0.51	
18	388	289	613	0.28	1.34	280	76	67	82	1.13	104	20	32	96	0.63	
Mean	377	317	831	0.33	1.20	480	160	127	153	1.27	102	19	37	92	0.51	
Group 3																
21	326	236			1.22		229	184	177	1.24		33	60	107	0.55	
22	378	291	466	0.27	1.30	563	199	148	167	1.34	111	26	39	97	0.67	
24	210	153			1.37		604	420	351	1.44		57	82	157	0.70	
25	132	119	238	0.31	1.11	1,260	486	333	347	1.40						
26	234	202			1.16		333	232	221	1.43		54	99	195	0.55	
28	133	144	286	0.37	0.92	727	306	201	198	1.52	143	23	70	141	0.33	
30	208	156			1.33		201	144	135	1.40		47				
35	100	120	346	0.35	0.83	505	207	141	175	1.62	104	21	47	126	0.45	
Mean	220	180	334	0.33	1.16	764	308	217	212	1.43	119	37	66	137	0.54	
Group 4																
40	72	62	91	0.26	1.16	687	282	183	213	1.49	161	48	62	182	0.79	
41	68	57	94	0.28	1.19	1,000	415	270	308	1.57	320	102	125	307	0.82	
42a	23	28	86	0.35	0.82	535	219	146	200	1.50	226	68	105	222	0.64	
46	48	49	109	0.29	0.98	1,236	541	333	376	1.62	268	97	102	226	0.95	
50	64	62	125	0.31	1.03	1,295	520	343	347	1.53	155	49	76	164	0.64	
52a	115	104	148	0.24	1.11	342	135	96	107	1.41	128	26	57	139	0.46	
53	40	35			1.14		346	255	181	1.36		81	175	288	0.46	
54	60	48			1.25		382	251	228	1.52		85	137	275	0.62	
55	61	65	146	0.30	0.94	458	185	122	156	1.51	139	30	55	155	0.55	
Mean	63	59	114	0.29	1.05	793	332	218	239	1.51	200	63	92	210	0.67	

* For abbreviations, see footnote to Table II.

† The values in parentheses are approximations based on serum triglyceride values.

‡ The addition of a letter to a serum number indicates a different but similar serum from the same patient. The TL values here are calculated as milligrams of dichromate reduced divided by 17.7.

creased in Group 2, and more substantially increased in Groups 3 and 4.

The composition of this fraction varied. Ratios of TC/PL ranged from less than normal values in Group 1 to greater than normal values in Group 4. Concentrations of total lipid instead of triglyceride of this and the D 1.063–1.21 fractions are presented because of the large errors

that may occur in calculating values of the relatively small TG component by difference. In cases of lactescent sera, it appeared that TG was increased relative to the other lipids of these fractions.

D 1.063–1.21 lipoproteins. The high density lipoproteins were below normal in Groups 1, 2 and 3, and above normal on average in Group 4. Ratios of TC/PL in the fraction were greater than normal, particularly in Group 4. The high cholesterol values in a few of the sera of Group 4 suggest the possibility of incomplete separation of lipoproteins of lower density.

D > 1.21 "lipid phosphorus." The quantity of ethanol-acetone soluble phosphorus remaining in the infranatant solution after centrifugation of the sera at D 1.21 varied considerably within each group, with no consistent difference from one group to another. The values in terms of phospholipid (P × 25) ranged from 5 to 25 mg per 100 ml of serum with a mean of 15 mg per 100 ml, compared with a normal range of 15 to 27 mg per 100 ml (11). The infranatant solution in the few cases analyzed was found to contain less than 3 mg of cholesterol per 100 ml of serum.

Summarizing the principal lipoprotein alterations in the different groups of sera with increased lipids, the lipoproteins of D < 1.019 were excessive in Group 1, both the D < 1.019 and the D 1.019–1.063 fractions were moderately increased in Groups 2 and 3, and principally the D 1.019–1.063 fraction was increased in Group 4. The D 1.063–1.21 fraction was diminished in Groups 1 through 3. It is evident that the previously discussed differences in serum triglyceride and in TC/TG (and also in TC/PL and PL/TL) in the different groups of sera were related to these differences in pattern of lipoproteins.

Different patterns of lipoproteins were also observed among the sera with slightly increased lipids (listed in Table III), as might be expected from the differences in TG levels and TC/TG ratios. The very low density lipoproteins were considerably above normal in serum no. 59. In most of the other sera, however, the lipoprotein abnormalities were not impressive.

Very low density lipoproteins and lactescence. The D < 1.019 lipoprotein fraction which included a broad spectrum of lipoproteins was divided in some cases into D < 1.006 and D 1.006–1.019 subfractions. The D < 1.006 portion contained the lactescent material, and the degree of lactescence of the sera was related to the quantity and triglyceride content of this portion. Results of analyses of lipids in the two subfractions are shown in Table V. The protein content of the D < 1.006 fraction was very low in proportion to lipid, considerably lower than that of the D 1.006–1.019 fraction, particularly in cases of lactescent sera. Lactescence appeared to be a result of the presence of lipoproteins of very low density which were high in triglyceride and low in protein.

TABLE V

Data on very low density lipoprotein fractions *

Serum no.	Serum lactescence	D < 1.006			D 1.006–1.019		
		TC	PL	TG	TC	PL	TG
		mg/100 ml of serum					
5a	++++	629	503	2,351	26	32	71
9	+++	366	339	1,047	20	22	38
12	+++	360	236	876	46	68	116
13	++	227	208	573	17	14	18
15	++	185	177	458	52	45	64
18	++	341	244	518	47	45	95
22	+	257	199	373	121	92	93
59	+	118	125	352	24	23	33
28	+	89	104	237	44	40	49
35	+	51	72	270	49	47	76
40	−	53	47	74	19	15	17
xx†	−	32	28	54	179	148	109
52a	−	42	36	63	73	68	85

* For abbreviations, see footnote to Table II.
† This serum was obtained during an acute infection and is not included elsewhere.

Factors influencing lipoproteins

It is evident from data already presented that the quantity of serum lipoproteins and the lipoprotein pattern (variations of which were reflected in differences in serum TG and TC/TG) bore a relation to the degree of hypoalbuminemia which in general was a good index of the severity of the nephrotic syndrome. The lipoprotein pattern in particular was also influenced by other factors, as is indicated by the irregular relationship between triglyceride and albumin. There were relatively great day to day variations in triglyceride values even when conditions were apparently stable. The lipid relationships in some cases were altered by hospitalization without spe-

cific therapy or changes in the disease. Changes in diet, glucose infusions (22), albumin infusions (23), or steroid therapy often resulted in changes in concentrations of individual lipids and in lipoprotein pattern. A large decrease in all lipids, particularly in triglyceride, occurred within 48 hours in one case, during an acute infection complicated by vomiting and diarrhea. Several elderly patients with nephrosis complicated by other diseases (especially malignancies) had unusually low levels of lipids. An attempt was made to eliminate, in so far as possible, the effects of these various complicating factors in the present study.

Lipoprotein pattern seemed to differ somewhat from patient to patient, presumably due to differences in endocrine and metabolic regulation, even when the disease state and the environmental factors discussed above were similar. The same pattern recurred during successive relapses of the disease in some individual patients, and the patterns often remained relatively constant for periods of weeks or months. The patterns, of course, changed from time to time in a majority of the cases, usually in relation to changes in the disease but sometimes without apparent cause. The magnitude and pattern of the lipid alterations often changed as renal insufficiency developed, possibly as a result of the decrease in proteinuria.

DISCUSSION

It is generally recognized that hyperlipidemia of considerable degree occurs more consistently in nephrosis or in the nephrotic syndrome than in types of renal disease not associated with marked proteinuria or hypoalbuminemia. From this and other evidence (24) it has been suspected that the lipid disturbance in nephrosis may be causally related to the deficiency of albumin, though some contrary evidence has been presented by Heymann, Nash, Gilkey and Lewis (25). The question is complicated by the irregular relationships among the individual lipids themselves, and even if deficiency of albumin is an important cause of the lipid disturbance, it may be that deficiency of other proteins of relatively small molecular size and possibly other entirely different disturbances also play a part. The present observations confirm the existence of a relationship between the levels of the serum lipids and serum albumin in nephrosis but indicate no more than the *possibility* that the hyperlipidemia is caused by hypoalbuminemia.

As already noted, levels of triglyceride were related irregularly to those of cholesterol and phospholipid, reflecting differences in lipoprotein pattern among the different sera. Total serum cholesterol and phospholipid were increased whenever albumin was decreased but triglyceride was regularly increased only when levels of serum albumin were very low. It is evident from these observations that the mechanisms regulating the concentrations of triglyceride were not identical with those governing the other lipids.

Serum triglyceride level was a good index of the quantity of very low density lipoproteins in the serum, and, in accord with the observations of Albrink, Man and Peters (26), was correlated with the degree of lactescence of the serum. Total serum cholesterol was not always an equally good index of the quantity of D 1.019–1.063 lipoproteins, which normally contain the major portion of serum cholesterol, because of the large amount of cholesterol sometimes present in the $D < 1.019$ lipoprotein fraction. Reasonably accurate estimates of the lipoprotein pattern could be made from a knowledge of both cholesterol and triglyceride levels, or from cholesterol level and the appearance of the serum, but not from cholesterol level alone.

Rosenman, Byers and Friedman (27), from studies on experimental nephrosis in rats, postulated that an accumulation of triglyceride due to a disturbance in the clearing mechanism is the primary lipid alteration in nephrosis, and that cholesterol and phospholipid accumulate passively as a result of trapping by the triglyceride. Lipoproteins were not studied. It appeared in the present study that an increase in cholesterol often occurred in the absence of any significant increase in triglyceride. Evidence of a decreased rate of conversion of S_f 10 to 400 lipoproteins to those of higher density (S_f 3 to 9) was observed in children with nephrosis by Gitlin and associates (28). This mechanism might explain in part the occurrence of sera of the Group 1 type with increased $D < 1.019$ lipoproteins, but other (perhaps earlier) disturbances must be present to account for increased levels of cholesterol, phos-

pholipid, and D 1.019–1.063 lipoproteins in sera such as those of Groups 3 and 4. Knowledge of the biochemical basis of these disturbances is scant.

The composite picture of the lipoprotein alterations in nephrosis which is suggested by this study may be summarized as follows. When nephrosis of mild to moderate degree develops, there is an increase in lipoproteins of D 1.019–1.063 and consequently in serum cholesterol and phospholipid, often accompanied by little change from normal in D < 1.019 lipoproteins. As the syndrome becomes more severe, the quantity of D < 1.019 lipoproteins usually becomes progressively greater and is reflected in an increase in serum triglyceride and lactescence. At the same time, the D 1.019–1.063 lipoproteins fall toward or even below normal. Serum cholesterol and phospholipid continue to increase, but come to be present principally in the D < 1.019 instead of the D 1.019–1.063 fraction.

The reason for the inverse relationship between quantities of the D < 1.019 and the D 1.019–1.063 fractions is not clear. A decrease in the D < 1.019 fraction and a simultaneous increase in the D 1.019–1.063 fraction have been observed to occur early in remissions of the disease induced by steroid therapy and during the course of repeated albumin infusions. Reciprocal changes in the fractions in the opposite direction sometimes occurred during prolonged infusions of glucose (22). The reciprocity between the fractions probably is not limited to nephrosis but is seen also in some other diseases. The decrease in one fraction as the other increased suggested the possibility of an interaction between lipoproteins of different densities in the serum. The relationship between the fractions seems to be metabolic, however, for in preliminary studies no change in quantity or composition of the fractions was noted when D < 1.019 and D 1.019–1.063 lipoproteins were mixed in various proportions and again separated at a density of 1.019 (29).

SUMMARY

1. Sera obtained periodically from 44 hospitalized patients with uncomplicated nephrosis (nephrotic syndrome) were analyzed for albumin, lipids, and, in many cases, lipoproteins.

2. Concentrations of total serum cholesterol, phospholipid, triglyceride, and total lipid were related inversely and nonlinearly to serum albumin levels. The relationship between triglyceride and albumin was less regular than that of the others, and the form of the relationship was different.

3. Pattern as well as total concentration of lipoproteins differed among the different sera with increased lipids. Either or both of the D < 1.019 and the D 1.019–1.063 lipoprotein fractions were abnormally large; however, these two fractions usually were not greatly increased simultaneously. Principally the D 1.019–1.063 lipoproteins were increased in a majority of cases of nephrosis without great deficiency of serum albumin. Severe nephrosis usually was characterized by high levels of the D < 1.019 lipoprotein fraction, often with near normal D 1.019–1.063 lipoproteins and subnormal high density (D 1.063–1.21) lipoproteins. Abnormalities were also noted in composition of the lipoprotein fractions.

4. Serum cholesterol and phospholipid were increased whenever the lipoproteins were increased, since both the D < 1.019 and D 1.019–1.063 lipoprotein fractions were rich in these lipids. Serum triglyceride and lactescence were increased appreciably only in sera with increased quantities of D < 1.019 lipoproteins.

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CORRECTION

In the paper entitled "Water and Electrolyte Studies in Cholera" by Watten, Morgan, na Songkhla, Vanikiata and Phillips (J. clin. Invest. 1959, **38**, 1879), the osmolarity values in Tables II and III were erroneously "corrected" according to Eisenman's formula on page 1881 of the paper.

EVALUATION OF THE SLOPE–VOLUME METHOD AS AN INDEX OF PULMONARY BLOOD VOLUME*

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The Stewart-Hamilton method (1) for measurement of blood volumes from indicator dilution curves has been validated in both theory (2) and practice (3), provided that certain conditions are met.

Measurement of the pulmonary blood volume by the Stewart-Hamilton method would entail injection of indicator into the main pulmonary artery and sampling from the left atrium. However, while insertion of a catheter into the left atrium of the dog is technically feasible, an unknown portion of the left atrial volume will be included in the measurement. A more serious objection is the evidence that the process of mixing of dye with blood in the left atrium is incomplete (4, 5), and thus one of the conditions for valid application of the Stewart-Hamilton method is not fulfilled. The interposition of the left ventricle and aortic valve is essential to achieve uniform cross-sectional distribution of indicator at the sampling site (6, 7). Therefore, it is necessary to place the tip of the sampling catheter in the aortic root distal to the aortic valve. The blood volume between the site of injection (main pulmonary artery) and the sampling site thus comprises both lungs and the chambers of the left side of the heart. This is probably the nearest approximation to the pulmonary blood volume that can be measured at present by the method.

Newman, Pearce and associates (8, 9), using a different analysis of indicator dilution curves, introduced the concept that the "slope volume" provides a measurement or index of the pulmonary blood volume. The slope volume is a function of the blood flow and the rate of diminishing concentration of the indicator during the phase between the peak concentration and commencing recirculation, hereinafter referred to as the "disappearance slope." The concept of slope volume has been derived from both theoretic reasoning and from demonstrations in model systems which have shown that, in a series of perfect mixing chambers, the largest single chamber will dominate the disappearance slope of the dilution curve, the other chambers influencing this slope only in the initial phase. The original assumption of Newman and his co-workers was that the lungs and heart chambers constitute such a series and that, therefore, the lungs, which presumably have the largest blood volume, should dominate the disappearance slope of the indicator dilution curve. More recently, Emanuel, Lacy and Newman (10) have defined the slope volume as "a volume of blood which would produce the exponential slope obtained if the flow mixed with all of the volume as if the volume were contained in a single completely mixed chamber (or a chamber made of parallel channels, all having the same ratio of flow to mixing volume)."

In the Stewart-Hamilton method for deriving the blood volume of the lungs and the left side of the heart, changes in pulmonary blood volume might well be obscured by simultaneous but opposite changes in blood volume in the left side of the heart. The slope volume, however, by providing an estimate of the blood volume of the lungs alone, might be expected to avoid such uncertainties. Therefore, the present studies were designed to test the validity of the slope volume as an index of the volume of blood in the lungs.

METHODS

Sixteen mongrel dogs weighing 14 to 25 kg were used. In 11 trained dogs, cardiac catheters were introduced under local anesthesia and in the remainder, under sodium pentobarbital anesthesia. A catheter, used for

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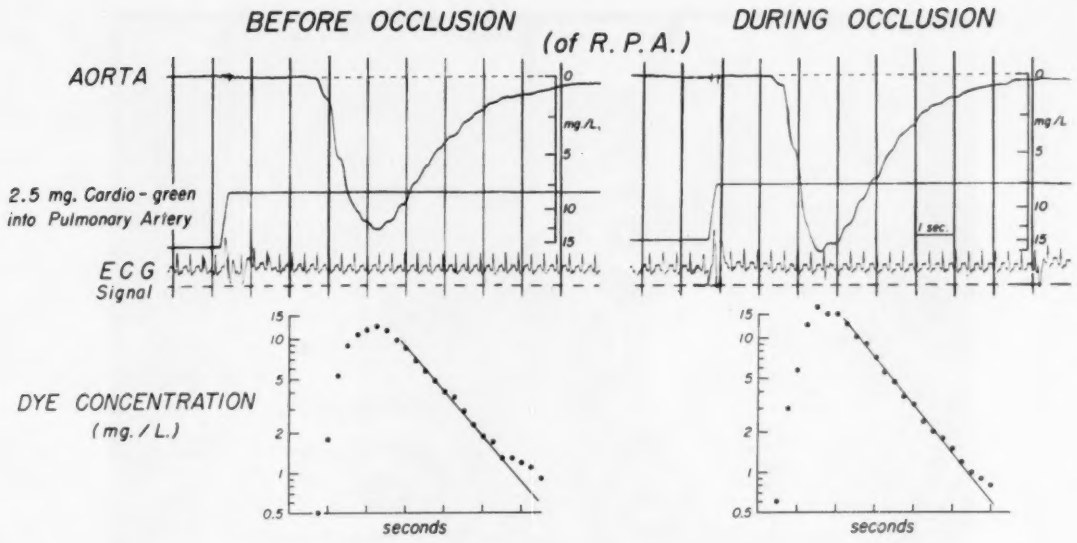

CARDIAC OUTPUT	4.4 L./min.	4.2 L./min.
MEAN TRANSIT TIME	4.4 seconds	3.4 seconds
LUNG and LEFT HEART BLOOD VOLUME	320 ml.	235 ml.
SLOPE VOLUME	92 ml.	85 ml.

FIG. 1. UPPER PANEL. INDICATOR DILUTION CURVES BEFORE AND DURING OCCLUSION OF THE RIGHT PULMONARY ARTERY IN A DOG WEIGHING 19.8 KG. The recirculation portions of the curves are not included. The deflection of the syringe line indicates the instant and magnitude of the injection. Note the shortening of the mean transit time during arterial occlusion.

LOWER PANEL. CURVES REPLOTTED ON A TIME AND LOG CONCENTRATION SCALE TO SHOW THE CLOSE SIMILARITY OF THE DISAPPEARANCE SLOPE OF BOTH CURVES.

injections of the indicator, was introduced via the external jugular vein into the pulmonary artery so that its tip lay just distal to the pulmonary valve. A 40 cm catheter having a dead space of 0.4 ml was inserted into the left ventricle via the carotid artery and withdrawn until its tip lay just above the aortic valve; this was used for sampling. The positions of the catheter tips were checked by fluoroscopy and manometry during introduction and at intervals throughout the observations. In a group of separate studies, the femoral artery was used as the sampling site. In 2 dogs, an additional no. 5 French catheter with a balloon about 4 to 6 cm from the tip was introduced into the pulmonary artery. The catheter was advanced until its tip became impacted in a distal branch; in this way, the position of the balloon remained fixed. The balloon could be inflated to occlude the right or left pulmonary artery or lobar branches thereof. The effectiveness of such occlusion was confirmed by angiograms made after injection of a 70 per cent solution of sodium acetrizoate (Urokon Sodium).

An indocyanine dye (cardio-green [1]) was used as the indicator at a concentration of 1.25 mg per ml in doses of 1.5 to 2.0 mg. Particular attention was paid to the characteristics of the injecting, sampling and recording systems, since determination of blood volume by the Stewart-Hamilton method depends especially on accurate measurement of the time components of the indicator dilution curve. Injections were made by a pneumatic syringe capable of delivering 1.0 ml of dye solution in 0.10 second. An automatic signaling device recorded the instant and volume of each injection (Figure 1). The dead space of the catheter and syringe system was kept filled with dye between successive injections. A densitometer [2] having a dead space of 0.1 ml was connected to the aortic catheter which had a dead space of 0.4 ml. When the method of Fox, Sutterer and Wood (11) for assaying dynamic response characteristics was

[1] Kindly supplied by Hynson, Westcott and Dunning, Baltimore, Md.

[2] This densitometer was developed by the Section of Engineering of the Mayo Clinic. It is similar to Model XC-100A manufactured by the Waters Corporation, Rochester, Minn., but it has a slightly larger internal diameter to permit high flows. The light-sensitive element is a phototube with an extremely high internal resistance. A power amplifier (a cathode follower) is used to obtain current, which is fed into a recorder.

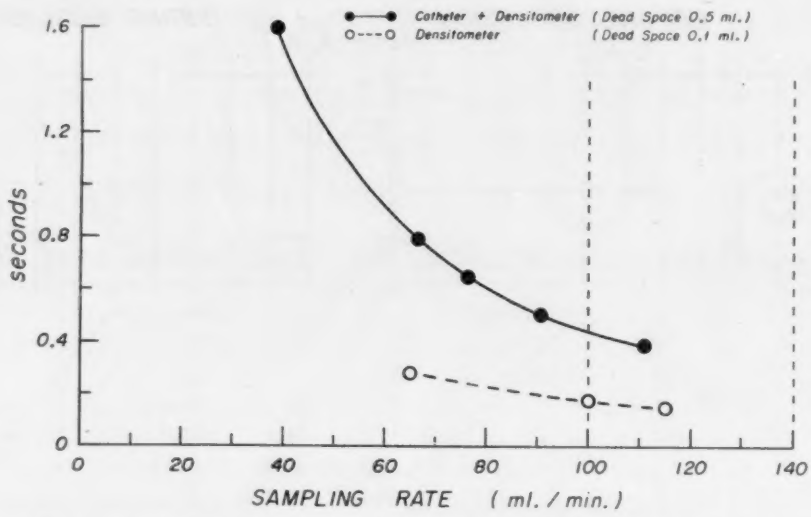

FIG. 2. DYNAMIC RESPONSE OF CATHETER-DENSITOMETER-GALVANOMETER SYSTEM. The time required to attain 90 per cent response to a "square wave" of dye (cardio-green) in blood is plotted against the sampling rate.

used, this densitometer-galvanometer system responded to a "square wave" input of cardio-green dye in blood by reaching 90 per cent of its maximal deflection in 0.20 second at the sampling rates of 100 to 120 ml per minute used throughout the studies (Figure 2). These sampling rates were achieved by use of vacuum suction at about 1.5 kg per square centimeter. A time correction was made in each instance for the mean transit time of indicator through the sampling system at the particular sampling rate used.

The indicator dilution curves obtained by this system show step functions (Figure 1). These are synchronous with the heart beat and represent changes in the concentration of dye at the aortic root during successive ejections of the left ventricle. Such curves contrast with the smoothly contoured curves obtained by means of sampling and recording systems that have slower dynamic response characteristics.

The volume between the injection and sampling sites was calculated by the Stewart-Hamilton formula (1), and the slope volume was determined by the method of Newman and co-workers (8). Calibration curves were obtained for each dog by drawing various known concentrations of dye in blood through the densitometer. In many experiments, dilution curves were obtained for blood at the start of the experiment (free of dye) and at the end of the experiment (with residual background dye). The calibrations usually were slightly different, as would be expected. However, the difference was never great, since cardio-green disappears from circulating blood much more quickly than does T-1824. When a difference was present, the calibrations were interpolated for intermediate dilution curves.

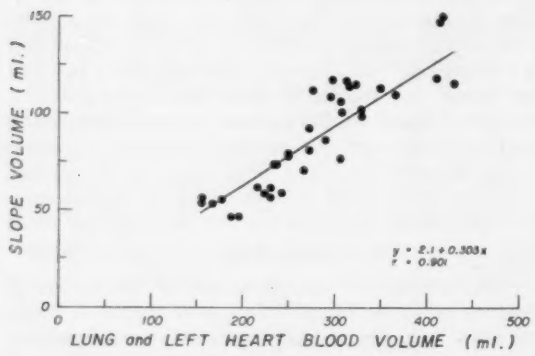

FIG. 3. SLOPE VOLUME PLOTTED AGAINST CORRESPONDING VOLUME OF BLOOD IN LUNGS AND LEFT SIDE OF HEART (36 DETERMINATIONS IN 16 DOGS). Note the correlation but the large systematic difference.

RESULTS

The pulmonary artery was used as the injection site and the aortic root as the sampling site in 36 determinations in the 16 dogs. Figure 3 shows the blood volumes, as calculated by the Stewart-Hamilton method, plotted against the corresponding slope volumes. There is a correlation ($R = 0.9$) but also a large systematic difference. The combined volume in the lungs and left side of the heart averaged 156 ml (SE 3.9) per 10 kg of body weight, whereas the slope volume averaged 48 ml (SE 1.7) per 10 kg of body

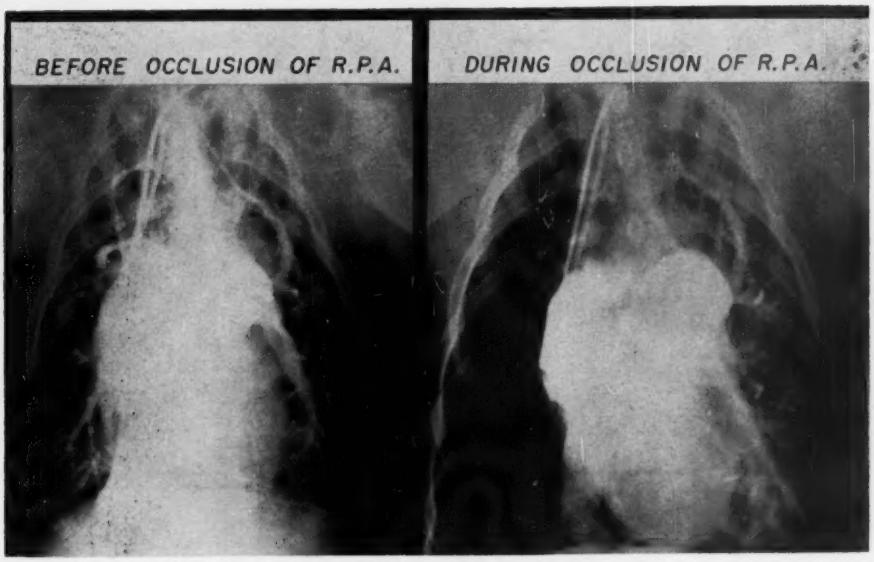

FIG. 4. ANGIOGRAMS AFTER INJECTION OF A 70 PER CENT SOLUTION OF SODIUM ACETRIZOATE INTO THE RIGHT ATRIUM. Before occlusion of the right pulmonary artery, the pulmonary vessels of both lungs are clearly visualized. During occlusion, there is no filling of the vessels of the right lung.

weight. It is of interest that Dow (12) found a practically identical ratio, namely 0.31, between slope volume and central volume in dogs. Thus, the slope volume is about 30 per cent of the blood volume in the lungs and left side of the heart. Some data obtained in our laboratory suggest that the lungs account for about two-thirds of the combined volume (13). This would make the slope volume about 45 per cent of the pulmonary blood volume.

These volumes were compared with nine determinations in seven dogs in which the femoral artery was used as the sampling site. Under these circumstances, the volume by the Stewart-Hamilton method averaged 227 ml (SE 15.9) per 10 kg of body weight, while the slope volume averaged 50 ml (SE 2.3) per 10 kg of body weight. The increase in the former volume was consistent with the different extent of the vascular bed included between the injection and sampling sites (13). The slope volume, however, was strikingly constant, a finding previously reported by Pearce, McKeever, Dow and Newman (9).

As already noted, the volume of the pulmonary vascular bed in two dogs was acutely and intermittently reduced by inflation of a balloon in the pulmonary artery. In one of these animals, the right pulmonary artery was occluded, the com-

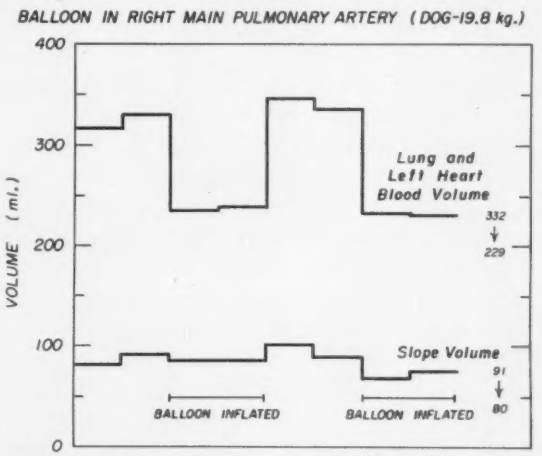

FIG. 5. CHANGES IN SLOPE VOLUME AND IN VOLUME OF BLOOD IN LUNGS AND LEFT SIDE OF HEART WITH INTERMITTENT OCCLUSION OF RIGHT PULMONARY ARTERY BY BALLOON-TIPPED CATHETER. The occlusion in each instance lasted from 7 to 10 minutes. In both this figure and in Figure 6, note the pronounced and reproducible changes in the blood volume of the lungs and left side of the heart in contrast to the incommensurate changes in slope volume.

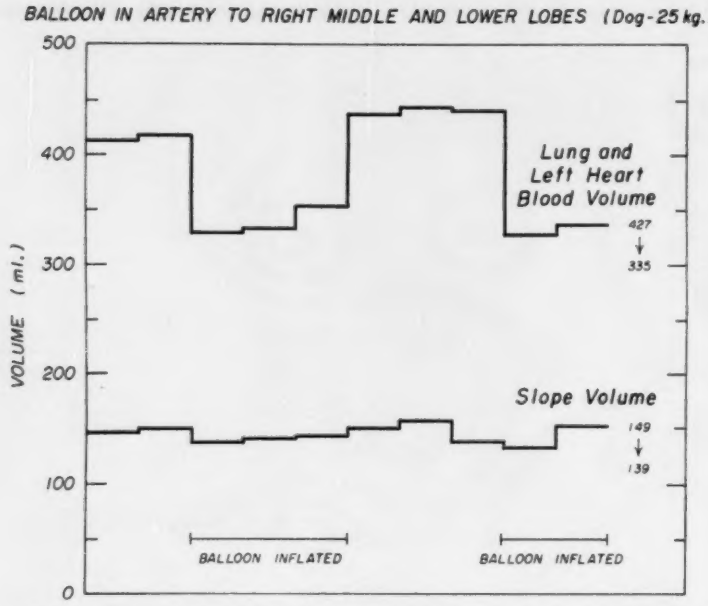

FIG. 6. CHANGES IN SLOPE VOLUME AND IN VOLUME OF BLOOD IN LUNGS AND LEFT SIDE OF HEART WITH INTERMITTENT OCCLUSION OF ARTERIES TO LOWER HALF OF RIGHT LUNG. The occlusion in each instance lasted from 7 to 10 minutes.

pleteness of the occlusion being confirmed by angiography (Figure 4). During occlusion, the cardiac output either remained unchanged or showed a modest decrease. These results are similar to the findings of Brofman (14) in normal man. However, the mean transit time of the indicator was reduced by about one-third (Figure 1). Since the volume of blood in the lungs and left side of the heart is the product of the cardiac output and the mean transit time, this volume was reduced by about one-third, the average volume decreasing from 332 to 229 ml (Figure 5). Volumes measured immediately on occlusion and five minutes later were similar. However, the slope volume was only slightly affected by the occlusion, the average volume decreasing from 91 to 80 ml (Figure 5). In the other dog, the lobar arteries to the lower half of the right lung were occluded, as confirmed by angiography. There was a corresponding decrease in the volume of blood in the lungs and left side of the heart of about one-fifth (427 to 335 ml), again with no consistent change in the slope volume, the average of which decreased from 149 to 139 ml (Figure 6). The procedure was repeated many times and yielded reproducible results (Figures 5 and 6).

DISCUSSION

The Stewart-Hamilton method depends on good mixing of the indicator with blood at the injection and sampling sites and also on constant flow during the period of observation. The first of these requirements is best fulfilled by injecting into the pulmonary artery and sampling from the aortic root (6, 7), as was done in these studies. The second requirement is not fulfilled, since flow through the aortic valve is not constant but pulsatile. That this theoretic objection may not be important in practice is suggested by the recent work of Schlant and associates (3), who found close agreement in measurement of the "central blood volume" in dogs by the Stewart-Hamilton method and by a technic using erythrocytes labeled with radiochromium (Cr^{51}).

The Stewart-Hamilton method makes no assumptions regarding the nature of blood flow through the lungs and is, therefore, valid whether such flow be laminar or turbulent. In contrast, the slope-volume method is based on the proposition that the lungs and heart chambers are a series of perfect mixing chambers. It is doubtful if the lungs can be so regarded (15–17). Theoretically,

movement through a capillary bed such as that of the lungs provides random scattering and longitudinal dispersal of the particles of dye rather than mixing. Sheppard and Savage (15) and Sheppard (16) have shown that the problem is more akin to the "random walk." Only when the dispersing effect is not too great can an approximately log-normal distribution in time of the outflowing dye be predicted, and this condition does not obtain in the large pulmonary capillary bed. Parrish, Hayden, Garrett and Huff (17) studied pulmonary transfer characteristics with radioisotope dilution technics, using an analog computer in such a way that both transport delay and dispersion could be varied independently. They found that the vessels of the lungs behave as a system with linear-flow and not as a mixing pool. Thus, it appears that the lungs cannot achieve the perfect and instantaneous mixing that is the chief assumption on which the slope-volume method is based.

It has been proposed recently that the increase in slope volume in mitral regurgitation indicates an increase in pulmonary blood volume or "effective mixing volume" (10). However, in mitral regurgitation, in addition to an enlarged upstream chamber, a to-and-fro flux of dye particles occurs across the incompetent valve, which in itself can spread the indicator dilution curve, decrease the disappearance slope, and thus increase the slope volume (18). The fact that the disappearance slope can be plotted semilogarithmically to yield a straight line is no guarantee that the disappearance slope is a single exponential function (19). It has become clear, through the use of sampling and recording systems with high dynamic response characteristics, that indicator dilution "curves" show step-like alterations in concentration corresponding to successive ventricular ejections (Figure 1), particularly when a central sampling site is used (20). Thus, when points are replotted on a semilogarithmic scale at short intervals of time, such as 0.5 second, they do not fall along a straight line, although the general trend is linear. In the present experiments, we constructed the best possible rectilinear fit through these points, designating this as the "slope." The absence of a completely smooth slope, which becomes evident when systems with high dynamic response characteristics are employed, is in itself an objection to the use of this parameter as a measure or an index of an intravascular volume.

As already noted, under the conditions of our studies the slope volume is about 30 per cent of the blood volume of the lungs and left side of the heart. Previous observations have suggested that about two-thirds of the blood volume between the pulmonary and aortic valves is contained in the lungs. Thus, as mentioned earlier, the slope volume is probably about 45 per cent of the total pulmonary blood volume. When the sampling site was changed from the aortic valve to the femoral artery, only slight changes in the slope volume were observed that were incommensurate with the changes in actual volume included between the two sites. These results could be interpreted as supporting the contention that the slope volume measures a volume directly related to the pulmonary blood volume. If so, a pronounced reduction in the slope volume would be anticipated after occlusion of one of the pulmonary arteries or branches thereof. However, such a reduction could not be demonstrated. It might be held that occlusion of the right pulmonary artery resulted in redistribution of blood, so that the left lung now contained an increased quantity of blood; this was not borne out by the concomitant Stewart-Hamilton calculations, which showed consistent reduction in this volume during periods of occlusion.

Thus, the slope volume neither gives a measurement of the volume of blood within the lungs nor consistently reflects the magnitude of changes induced therein.

SUMMARY

Studies were carried out to test the validity of the Newman slope-volume method as an index or measurement of the volume of blood in the lungs. Indocyanine (cardio-green) dye was injected into the pulmonary artery of the dog, and indicator dilution curves were inscribed at the aortic root using a densitometer with a high frequency response. Sampling was from the aorta rather than the left atrium to ensure adequate mixing of the dye with blood. The Stewart-Hamilton method was used to derive the volume of blood in the lungs and the chambers of the left

side of the heart; the Newman method was used to calculate the slope volume.

In 36 determinations in 16 dogs, the volume of blood in the lungs and left side of the heart was 156 ml (SE 3.9) per 10 kg of body weight, whereas the slope volume was 48 ml (SE 1.7) per 10 kg of body weight. Thus, the slope volume is about 30 per cent of the volume in the lungs and left side of the heart and it is probably about 45 per cent of the pulmonary blood volume.

With repeated temporary occlusion of the right main pulmonary artery, the volume of blood in the lungs and left side of the heart decreased from an average of 332 to 229 ml, while the average slope volume decreased from 91 to 80 ml. With occlusion of the arteries to the lower half of the right lung, the former volume decreased from 427 to 335 ml, while the latter decreased from 149 to 139 ml.

With the assumption that the Stewart-Hamilton method is valid, it appears that the slope volume neither gives a measurement of the volume of blood in the lungs nor reflects the magnitude of changes induced therein.

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CHROMATOGRAPHIC PROPERTIES OF GAMMA GLOBULIN: BEHAVIOR OF SERUM GAMMA MACROGLOBULINS *

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The cellulose ion exchangers (1) have proved to be useful chromatographic media for the serum proteins. In the fractionation of normal human serum on the anion exchanger, diethylaminoethyl (DEAE) cellulose, application of buffer gradients of increasing concentration and decreasing pH results in the appearance of components in the effluent fluid in order of increasing electrophoretic mobility (2), indicating that ion exchange is the predominant mechanism involved. In the case of the gamma globulin component, some subfractionation was demonstrated in terms of progressively increasing electrophoretic mobility of column effluents of this fraction. Little attention has been directed, however, to the chromatographic properties of the gamma globulins of high molecular weight, 19S or greater, which are found in large quantities in certain pathological sera, and also in small quantities in normal human sera (3).

In the course of anion exchange fractionation of sera from patients with rheumatoid arthritis, the "rheumatoid factor" (4–6), a gamma globulin constituent of high molecular weight (7), appeared in column effluents far removed from the bulk of the gamma globulin (8). This observation indicated that the chromatographic behavior of certain gamma globulins, and possibly of other serum proteins, on DEAE cellulose is governed not only by ion exchange but also by other factors which depend in part upon molecular size. Our attention was therefore directed to sera known to contain large amounts of abnormal gamma globulin constituents. The results obtained have been previously presented in abstract form (9).

MATERIALS AND METHODS

DEAE cellulose [1] (1 g for each 30 to 40 mg of protein fractionated) was equilibrated with 0.01 M phosphate buffer at pH 7. The equilibrated exchanger was then poured onto columns (2.5 × 15 cm) equipped with fritted glass filters, and packed under mild air pressure. Before chromatogarphy, sera were diluted with an equal volume of distilled water and dialyzed for two days against 3 to 6 L of 0.01 M phosphate buffer at pH 8.5 to 9. In one instance, the euglobulin precipitate from the serum of a patient with macroglobulinemia was fractionated. This was prepared by 15-fold dilution of 4 ml of the serum with cold water. The precipitate formed was harvested by centrifugation, washed four times with cold water, dissolved in 0.01 M phosphate buffer at pH 8.6 and chromatographed.

Chromatographic fractionation was carried out at room temperature using relatively rapid flow rates of 1 to 2 ml per minute as described previously (8). The protein content of each effluent fraction was determined by measurement of ultraviolet absorption at 280 mμ in the Beckman spectrophotometer. Readings were taken as fractions and were collected in order to follow the course of separation and to determine when a change in the eluting buffer was required. At the end of each chromatographic experiment, samples of each peak were concentrated 10- to 100-fold by ultrafiltration through Mies collodion bags [2] to a final protein concentration of approximately 1 per cent. Paper electrophoretic analysis was carried out in a Spinco (Durrum) apparatus at pH 8.6 ($\Gamma/2 = 0.075$) for 16 hours at 120 v and 4 ma. After staining with bromphenol blue, paper strips were analyzed in a densitometer.[3] Ultracentrifugal analyses were carried out in the Spinco Model E analytical ultracentrifuge at 59,780 rpm. The sedimentation constants reported were not corrected for concentration dependence.

Precipitation and elution of the rheumatoid factor were carried out as previously described (8).

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[1] Brown and Co., 500 Fifth Avenue, New York, N. Y.

[2] Schleicher and Schuell, Keene, N. H.

[3] Analytrol, Spinco, Palo Alto, Calif.

TABLE I

Patients with hyperglobulinemia

Patient	Diagnosis	Total serum protein	Serum albumin	Serum globulin	Serum gamma globulin
		g %	g %	g %	g %
M. S.	Liver disease	17	2.55	14.45	10.71
J. C.	Primary amyloidosis	8.2	2.59	5.65	3.36
M. K.	Multiple myeloma	17	2.55	14.45	10.71
C. R.	Multiple myeloma	8.6	3.0	5.60	5.56*
B. B.	Macroglobulinemia	11.2	3.57	7.64	5.75
S. K.	Macroglobulinemia	10.9	2.08	8.80	7.35

* Represents per cent beta globulin; gamma globulin content was 0.05 g per cent.

Rabbit antisera against normal human 7S and 19S gamma globulins (10) were used in capillary precipitin tests. The rabbit antiserum to 19S gamma globulin had been absorbed with human 7S gamma globulin prior to use. Both antisera were kindly provided by Dr. Edward C. Franklin of the Rockefeller Institute.

The sera [4] investigated were obtained from one normal individual and from patients with the following: lymphosarcoma, 1; liver disease [an atypical case of the syndrome described by Bearn, Kunkel and Slater (11)], 1; primary amyloidosis, 1; multiple myeloma, 2; and macroglobulinemia (Waldenström syndrome), 2. Pertinent laboratory data on the patients enumerated are given in Table I.

RESULTS

Three types of chromatographic pattern were obtained with the sera investigated. In one type, represented by a normal serum and sera of patients with ordinary hyperglobulinemia, substantially all of the gamma globulin was found in the first chromatographic peak eluted at pH 7. A second type, represented by a serum from a patient with beta myeloma, was characterized by elevation of an intermediate peak eluted at pH 6. In the third type, which was obtained with sera from patients with macroglobulinemia, the major peak, composed mainly of macroglobulins, was eluted at pH 5 at the tail end of the chromatograms.

The first type of pattern is illustrated by sera obtained from patients with primary amyloidosis (Figure 1) and multiple myeloma (Figure 2). In both instances, the serum gamma globulin concentration was high (Table I) and was eluted almost completely in the pH 7 peak. Little or no gamma globulin was found in subsequent chromatographic fractions (Figures 1 and 2, Table II). The same gamma globulin distribution was observed with a normal serum [5] and with the sera of one patient with lymphosarcoma and another with liver disease of the type described by Bearn, Kunkel and Slater (11). In all of these, the major portion of the gamma globulin was eluted in the pH 7 peak and was entirely of the 7S variety. The small amount of gamma globulin found

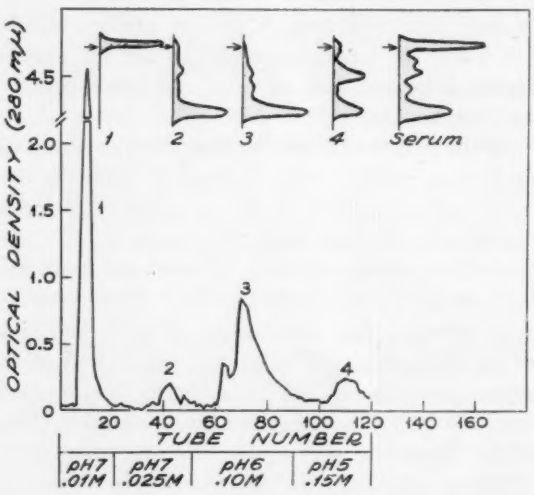

FIG. 1. CHROMATOGRAPHIC PATTERN OF SERUM FROM A PATIENT (J.C.) WITH PRIMARY AMYLOIDOSIS, AND PAPER ELECTROPHORETIC PATTERNS OF CHROMATOGRAPHIC FRACTIONS. Three ml serum on 6 g DEAE cellulose; 5 ml fractions.

[4] The sera of the patients with multiple myeloma (M.K.), lymphosarcoma (V.H.), and one with macroglobulinemia (S.K.) were kindly provided by Dr. Elliott Osserman of Columbia University. The other macroglobulinemia serum (B.B.) was obtained through the courtesy of Dr. Gerald Weissman of the Mt. Sinai Hospital, N. Y.

[5] Following submission of the manuscript of this paper, it was reported by Fahey and Horbett (12) that gamma globulin from normal individuals may be fractionated on DEAE cellulose into light and heavy components.

TABLE II

Ultracentrifugal analysis of chromatographic fractions containing gamma globulin, normal serum, and hyperglobulinemia sera

Patient	Diagnosis	Serum	Elution		Effluent volume	Sedimentation constant of gamma globulins S_{20w}	% Total protein of peak
			pH	Molarity			
		ml			*ml*		
K. S.	Normal subject	25	7	0.01	400	6.6*	100
V. H.	Lymphosarcoma	2	7	0.01	100	6.6	100
M. S.	Liver disease	2	7	0.01	80	6.2	100
J. C.	Primary amyloidosis	3	7	0.01	80	7.2	100
			5	0.15	100	17	4
M. K.	Multiple myeloma, γ type	2	7	0.01	60	6.2	100
C. R.	Multiple myeloma, β type	2	7	0.01	40	7.3	100
			6†	0.10	140	6.3	62
						9.1	11
						12	4
						14	3
			5†	0.15	100	6.2	12
						8.6	55
						10	23
						12	8
						14	2

* An 18S gamma globulin component was noted in the fraction eluted at pH 5 (0.15M) but was too small to be measured.

† The group of components eluted at this pH had the electrophoretic mobility of beta globulin.

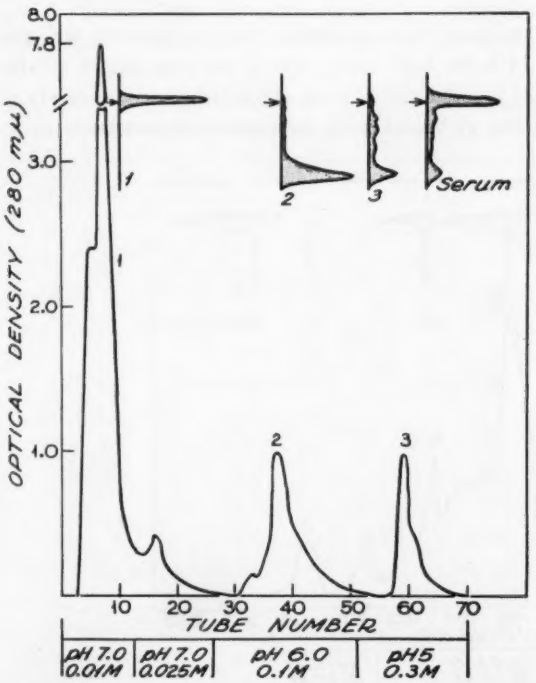

FIG. 2. CHROMATOGRAPHIC PATTERN OF MULTIPLE MYELOMA SERUM (M.K.) OF GAMMA TYPE, AND PAPER ELECTROPHORETIC PATTERNS OF CHROMATOGRAPHIC FRACTIONS. Two ml serum on 4 g DEAE cellulose; 5 ml fractions.

in two instances in the pH 5 peak was of the 19S type.

The second type of chromatographic pattern was obtained with the serum of a patient with beta multiple myeloma (Figure 3). The distinguishing feature of this pattern is the great elevation in the pH 6 peak. In this instance, the pH 7 (0.01) peak was relatively small and con-

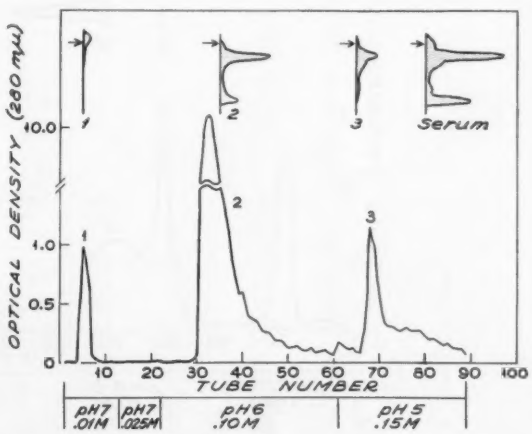

FIG. 3. CHROMATOGRAPHIC PATTERN OF MULTIPLE MYELOMA SERUM (C.R.) OF BETA TYPE, AND PAPER ELECTROPHORETIC PATTERNS OF CHROMATOGRAPHIC FRACTIONS. Two ml serum on 6 g DEAE cellulose; 5 ml fractions.

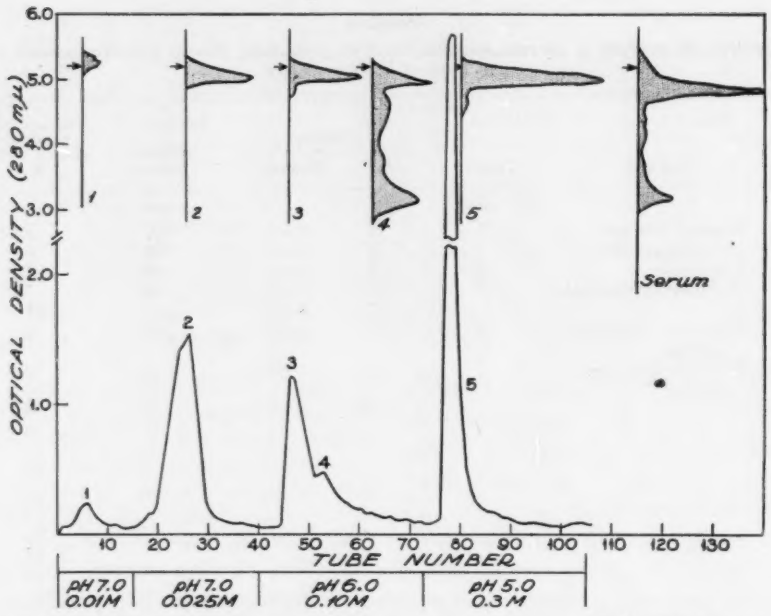

FIG. 4. CHROMATOGRAPHIC PATTERN OF SERUM FROM A PATIENT (B.B.) WITH MACROGLOBULINEMIA, AND PAPER ELECTROPHORETIC PATTERNS OF CHROMATOGRAPHIC FRACTIONS. Two ml serum on 5 g DEAE cellulose; 4 ml fractions.

sisted entirely of 7S gamma globulin. On ultracentrifugal analysis, the pH 6 peak contained five components, one of which was albumin and the rest beta globulins by paper electrophoresis. The sedimentation constants ranged between 6.3 and 14S, the 6.3S being the major component (Table II). The pH 5 peak consisted almost entirely of beta globulins with sedimentation constants rang-

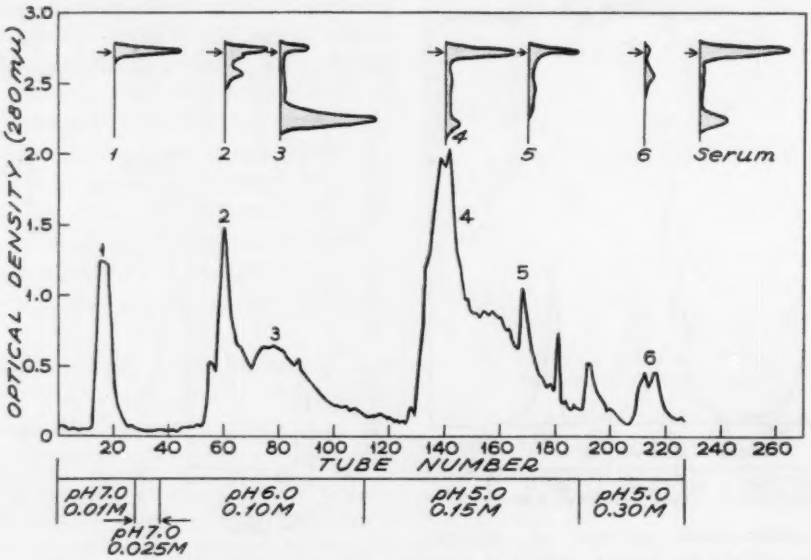

FIG. 5. CHROMATOGRAPHIC PATTERN OF SERUM FROM A PATIENT (S.K.) WITH MACROGLOBULINEMIA, AND PAPER ELECTROPHORETIC PATTERNS OF CHROMATOGRAPHIC FRACTIONS. Four ml serum on 10 g DEAE cellulose; 4 ml fractions.

TABLE III

Precipitation tests on chromatographic fractions using rabbit antiserum to 7S and 19S gamma globulin

Effluent fraction		Waldenström syndrome (Figure 4)		Rheumatoid factor, F II precipitate	
pH	Molarity	Anti-7S	Anti-19S	Anti-7S	Anti-19S
7	0.01	+++	0	+++	0
7	0.025	++	+		
6	0.10	+	+		
5	0.30	+++	++	+++	+++

ing from 6.2S to 14S. The major ultracentrifugal component, comprising 55 per cent of the total protein of this peak, had an S rate of 8.6S (Table II).

Chromatograms of the third type, demonstrated by the sera of patients with macroglobulinemia (Figures 4 and 5), are characterized by the occurrence of gamma globulins in each of the peaks and the presence of a large pH 5 peak composed mainly of gamma globulin. Ultracentrifugation of the whole serum of Patient B. B., of which 53 per cent of the total protein was gamma globulin (Table I), indicated the presence of four components, one of which had a sedimentation constant of 4S and three sedimentation constants of 8, 17, and 25S, respectively. The 17S component was the major component. Unlike those of the normal, multiple myeloma, and other hyperglobulinemic sera studied above, all chromatographic peaks, as mentioned, contained large amounts of protein with the electrophoretic mobility of gamma globulin. The protein of the pH 7 (0.01 M) peak was entirely gamma globulin, but too small in concentration to examine in the ultracentrifuge. It gave no reaction with an antiserum to 19S gamma globulin (Table III), but did react with an antiserum to 7S gamma globulin. The pH 7 (0.025 M) and pH 6 peaks consisted mainly of a 17S macroglobulin with small amounts of 8S and approximately 26S material. The pH 5 peak consisted mainly of an 18S component and was free of light gamma globulin.

A second serum from a patient with macroglobulinemia (S.K., Figure 5) contained 67 per cent gamma globulin. On ultracentrifugation, there were five components, one of which had a sedimentation constant of 4.7S, the others having constants of 6.7, 17, 25, and 31S, respectively. The 17S component was the major gamma globu-

TABLE IV

Ultracentrifugal analysis of chromatographic fractions containing gamma globulin, macroglobulinemia sera

Patient	Serum	Elution		Effluent volume	Sedimentation constant of gamma globulins S_{20w}	% Total protein* of peak
		pH	Molarity			
	ml			*ml*		
B. B.	2	7	0.01	40		
		7	0.025	60	8	17
					17	71
					27	12
		6	0.10	80	7.7	8
					18	81
					28	11
		5	0.3	64	18	60
					27	9
S. K.	4	7	0.01	100	6.3	100
		6	0.10	128	17	58
					24	7
		6	0.10	248	16	16
					25	5
		5	0.15	360	18	47
					24	21
					31	9
S. K. (Euglobulin fraction)	4†	5	0.15	70	18	92
					25	8
		5	0.15	130	18	70
					25	24
					31	6

* In those instances where the total is not 100 per cent, the difference represents non-gamma globulin components.
† The euglobulin fraction was prepared from this volume of serum.

lin component, making up 40.5 per cent of the total protein of the serum. The pH 7 (0.01 M) peak consisted entirely of gamma globulin with a sedimentation coefficient of 6.3S. The serum macroglobulins with sedimentation constants of approximately 18, 25, and 31S were found distributed over the pH 6 and pH 5 peaks (Table IV). Other serum proteins were also eluted in the pH 6 and pH 5 peaks; beta globulin (4.9S) and albumin (3.8S) were eluted in the pH 6 peak, and alpha globulins (3.6S) chiefly in the pH 5 peak.

Thus, in the case of the macroglobulinemia sera, which contained little low molecular weight gamma globulin, the abnormal, heavy components were distributed over a number of chromatographic peaks with the bulk of the gamma globulin appearing in the pH 5 peak, the last to be eluted. The first peak, eluted at pH 7.0, which contained 7S gamma globulin, was relatively small in size. This is in contrast to the results obtained with the serum of normal individuals, patients with rheumatoid arthritis (8), and the other hyperglobulinemic states described above.

A clear demonstration of the chromatographic behavior of the macroglobulins was obtained on chromatography of the euglobulin fraction from the serum of Patient S.K. The pattern obtained showed only two major components, both of which appeared in the pH 5 peak and consisted entirely of gamma globulin. On ultracentrifugal analysis (Table IV), one fraction contained two components with sedimentation constants of 18 and 25S, while the other contained three components with constants of 18, 25, and 32S. The relative amounts of these components in the second fraction, however, were different from those in the first fraction, a higher concentration of heavier components appearing in the second fraction.

A definitive separation of gamma globulins of differing size was obtained with sera of patients with rheumatoid arthritis. The presence in such sera of the rheumatoid factor, a 19S gamma globulin (7), offered a unique opportunity to study this phenomenon. The factor was first precipitated from the serum by addition of human Fraction II and the precipitate washed and dissolved in 4 M urea. The urea solution was then applied to the DEAE cellulose column and the pattern obtained (8) showed a distinct separation into two major peaks, eluted at pH 7 (0.01 M) and pH 5 (0.3 M), respectively, each having the electrophoretic mobility of gamma globulin. On ultracentrifugal analysis, the first peak had a sedimentation constant of 7S and the second consisted almost entirely of 19S gamma globulin with no detectable 7S protein (8). No contamination of the 7S with 19S gamma globulin was detectable by immunochemical testing (Table III). Rheumatoid factor activities including precipitation with Fraction II (13), agglutination of sensitized sheep cells (14), and agglutination of latex particles (15, 16) were found only with the second or 19S peak.

Immunological tests. Capillary precipitin tests performed on the chromatographic fractions obtained with the serum from a patient with macroglobulinemia (Figure 4) and from the precipitate between rheumatoid factor and Fraction II (8) are given in Table III. The rabbit antisera used (10) were: *1*) an antiserum to 7S gamma globulin, and *2*) an antiserum to 19S gamma globulin which, after absorption with 7S gamma globulin, reacts only with the macroglobulin. All of the fractions shown in Table III gave positive precipitin tests with the 7S antiserum as might be expected, since all contained gamma globulin. The presence of macroglobulins, however, is clearly demonstrated only in the pH 7 (0.025 M), pH 6, and pH 5 peaks of Figure 4 and the pH 5 peak of the rheumatoid factor-Fraction II precipitate by the positive precipitin reactions obtained with the antiserum to 19S gamma globulin.

DISCUSSION

The gamma globulin of normal human serum consists of at least four ultracentrifugally distinct components with sedimentation coefficients of 7, 19, 28, and 44S, respectively (3). The first two may be detected in unconcentrated serum and the latter two only after considerable enrichment. The experiments reported in this paper, a development of the chromatographic technique of Sober, Gutter, Wyckoff and Peterson (2), provide a basis for the separation of the 7S from the heavier gamma globulins. In the cases of normal sera; multiple myeloma sera; the sera from patients with liver disease, lymphosarcoma, and primary amyloidosis; and the precipitate between

Fraction II and the rheumatoid factor, it was possible to separate 7 and 19S gamma globulins in a single chromatographic step. The first peak obtained in each instance consisted entirely of 7S gamma globulin as shown both by ultracentrifugation and immunological tests. Beyond this point, little or no gamma globulin was eluted except for the small amount, presumably macroglobulin, present at the tail end of the normal serum chromatogram.

Although the two multiple myeloma sera studied contained considerable amounts of myeloma protein, these were eluted early in the chromatographic procedure. The protein of the gamma globulin type present in the serum of M.K. (Figure 2) was eluted in the pH 7 peak, while the myeloma protein of the beta globulin variety in the serum of C.R. (Figure 3) was eluted mainly in the pH 6 peak, which has usually contained the beta globulins. These results are consistent with those reported by Fahey, McCoy and Goulian (17), and with the observations that myeloma proteins are of the low molecular weight variety (18). In contrast, the patterns obtained with the two macroglobulin-rich sera (Figures 4 and 5) show a series of peaks of which those eluted last tend to be large and contain a large amount of gamma globulin. In this respect, the patterns differ markedly from those obtained with normal and ordinary hyperglobulinemic sera (Figures 1 and 2).

The ultracentrifugal analysis of the chromatographic fractions of the sera from patients with macroglobulinemia is of particular interest. In general, the pH 5 peak, the last to be eluted, contains high molecular weight gamma globulin but no gamma globulin of the 6S to 8S variety. Substantially all of the latter protein is found in the early peaks and usually in the pH 7 (0.01 M) peak, the first to be eluted. Analysis of the individual peaks from the macroglobulin-rich sera showed that the gamma globulin fraction of all of the peaks which contained macroglobulin consisted mainly of a 15 to 18S component with smaller amounts of heavier components.

The elution of mixtures of macroglobulins with a similar distribution of S rates in separate chromatographic peaks was observed in some experiments. Particularly striking is the comparison of the pH 7 (0.025 M) and the pH 6 (0.1 M) peaks of Patient B.B. (Figure 4, Table IV), which shows that the only significant difference in composition of the two peaks is the presence of a larger percentage of 7S gamma globulin in the pH 7 peak. On the basis of previously reported observations on the chromatography of human serum albumin (19), it is suggested that the proteins eluted in the pH 7 peak may represent macroglobulins present in excess of the binding capacity of the column at the buffer concentration used to elute this peak.

The mechanism responsible for the separation of the macroglobulins from 7S gamma globulins on DEAE cellulose is not clear. The tendency of the heavy proteins to appear in chromatographic eluates of higher salt concentration than those in which 7S gamma globulin is eluted, even when the pH of the eluting buffer is kept constant (8), may be dependent on a number of factors. Evidence against the possibility that solubility plays an important part is the observation that the chromatographic patterns of the macroglobulinemic sera were similar despite the fact that one serum (S.K.) gave a heavy euglobulin precipitate on dilution with water, while the other (B.B.) gave none.

The increased affinity of the high molecular weight gamma globulins for DEAE cellulose may be based upon at least two factors which differentiate these proteins from the 7S gamma globulins. One is their higher carbohydrate content (20), and the other their greater total charge. Since the 19S gamma globulins consist of six or more 7S molecules, the total charge on the macroglobulin would be greater than that of its individual 7S constituents. It is also possible that the high molecular weight gamma globulins have greater net charge, but because of differences in the shapes of the molecules and resultant differences in resistance to flow, the electrophoretic mobility is similar in magnitude to that of 7S gamma globulin. These properties could be responsible for both increased electrostatic and molecular binding. It is noteworthy that in experiments using the cation exchanger, carboxymethyl cellulose, 19S gamma globulin was bound so firmly that it could be eluted completely only after elution with 0.1 M sodium hydroxide. This suggests that non-ionic forces play an important role in the binding of the macroglobulins.

While it has been possible to separate 7S and 19S gamma globulins effectively by the technique described, little can be said about the separation from each other of the minor gamma globulin constituents of larger molecular size. Though fractionation of the 17S, 25S, and 30S components was achieved (Table IV), complete separation of these components from one another was not effected. It should also be mentioned that the difference in chromatographic behavior observed with gamma globulins of different size may eventually be demonstrable among the other serum proteins which are heterogeneous on ultracentrifugal analysis; i.e., the 5S and 12S beta globulins and 4S and 19S alpha-2 globulins (3).

SUMMARY

1. Gamma globulin has been separated on DEAE cellulose into components which differ in molecular size.

2. When sera from patients with liver disease, lymphosarcoma, primary amyloidosis, multiple myeloma, and macroglobulinemia were studied, the appearance of the chromatographic pattern of the serum on DEAE cellulose in conjunction with paper electrophoresis made it possible to detect the presence of increased amounts of macroglobulins. It has been possible in this way to differentiate between the chromatographic patterns of diseases with ordinary hypergammaglobulinemia (including multiple myeloma) and macroglobulinemia.

3. The chromatographic method employed offered a means of preparing 7S gamma globulin free of heavier molecular species. In the case of the rheumatoid factor, the 19S gamma globulin has been prepared free of the 7S variety.

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A STUDY OF DISTAL RENAL TUBULAR FUNCTIONS BY A MODIFIED STOP FLOW TECHNIQUE

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The stop flow technique has afforded a relatively simple method for the localization of certain renal tubular functions (1–3). However, as previously indicated by Pitts, Gurd, Kessler and Hierholzer (2), the method has certain technical shortcomings. As a result of contamination of initial specimens by preformed urine in the renal pelvis, admixture of specimens in the pelvis during outflow, and variations in nephron length, some lack of precision in localization is inevitable. In addition, water reabsorption in the distal tubular system may lead to displacement of the intratubular fluid column toward a more distal site (4), and consequently distort the various concentration patterns. This latter process is minimized, however, during mannitol diuresis, because of the limited movement of water out of the tubular lumen (5, 6). Finally, any specimen trapped within the tubule, proximal to the terminal collecting duct, may be modified during its subsequent passage through the nephron after release of ureteral occlusion. Consequently, accurate quantification of the movements of water and electrolytes, particularly in specimens derived from the proximal tubule, is impossible.

In the present study, attention has been focused on events which occur in the distal portion of the tubular system, the collecting ducts and the distal convolutions, during the period of stop flow. Experiments have been conducted during water, sodium chloride, sodium sulfate, and mannitol diuresis. This has permitted the testing of certain previously proposed hypotheses derived from stop flow experiments performed exclusively during mannitol diuresis. Investigation of the concentrating mechanism has also been possible, a procedure largely precluded in stop flow experiments conducted during hypertonic mannitol diuresis.

The dead space within the renal pelvis, comprising 2 to 3 ml of urine, considerably distorts the concentration patterns in specimens from the distal tubular segments. This is partially offset in experiments performed during mannitol diuresis because of the large volume of fluid (up to 20 ml) obtained prior to the appearance of new glomerular filtrate. Thus each tubular segment has a large volume representation in the collected outflow. During water or sodium chloride diuresis, however, in which the comparable volume is of the order of 3 to 7 ml, the pelvic dead space constitutes a major restriction to accurate studies. Consequently, a means of eliminating this dead space has been devised, and is utilized in the present experiments. In addition, a method for labeling fluid from the region of the distal convoluted tubule (analogous to the use of para-amino hippurate to tag proximal urine) has been utilized in order to obtain more precise localization of the various distal tubular functions. Data concerning the relative locations and interrelationships of sodium, chloride, potassium, and water movements have been obtained.

METHODS AND PROCEDURE

With the exception of the modifications to be described, the basic technique as first proposed by Malvin, Sullivan and Wilde was employed (7). All experiments were performed on female mongrel dogs, anesthetized with intravenous pentobarbital or thiopental. The latter was used in all experiments in which a water diuresis was produced (8).

The left ureter was catheterized through a flank incision. The pelvic dead space was obliterated by the following procedure. At the time of operation, the shaft of a short no. 23 needle, attached to a length of no. 50 polyethylene tubing (Intramedic), was inserted through the renal parenchyma into the kidney pelvis. The pelvic catheter was led out through the skin incision, which was closed with clips. Just prior to the time of occlusion the ureteral catheter was attached to a needle on a stopcock (stopcock no. 1 in Figure 1) and the urine was allowed to escape through the side arm. As shown in Figure 1, the other end of this stopcock (no. 1) was attached to an-

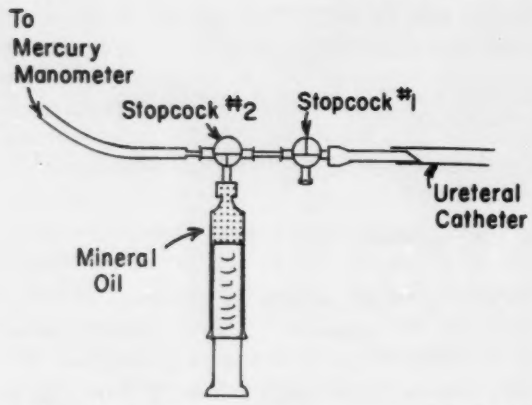

FIG. 1. APPARATUS USED FOR URETERAL OCCLUSION.

other stopcock (no. 2) which had been modified so that all three openings intercommunicated freely. To the side arm of stopcock no. 2 was attached a syringe filled with mineral oil, stained with Sudan Black. The distal opening was connected to a mercury manometer. Immediately prior to stopping flow, 20 ml of air was rapidly injected into the pelvic catheter and allowed to escape through the side arm of stopcock no. 1. This served to wash the urine out of the renal pelvis and ureteral catheter. Stopcock no. 1 was then turned so that the ureteral catheter communicated with stopcock no. 2, and mineral oil was injected from the syringe until a pressure of 100 to 110 mm of mercury was attained. This procedure was completed within five seconds of starting the air washout. Any residual air or urine within the renal pelvis was allowed to reflux through the pelvic catheter, which was clamped when only mineral oil was refluxing. The above pressure was maintained throughout the stop flow period, thus preventing entrance of newly formed urine into the pelvis from the kidney.

At the time the flow was started, the initial urine was collected by allowing it to pass serially into several lengths of no. 200 polyethylene tubing. Approximately the first 2.0 ml of urine was collected in this fashion. Subsequent 0.5 ml samples were collected in small test tubes. The initial urine was easily distinguished from the colored mineral oil, with which it was often admixed. Serial 0.17 ml specimens were removed from the tubing by measuring the proper fluid column lengths, clamping and cutting the tubing, and transferring the specimens to small plastic containers.

In order to localize the concentration patterns observed in relation to the distal convoluted tubule, an attempt was made to label the urine contained within the renal cortex during the occlusion period. A small (no. 23) needle tip connected to a length of no. 50 polyethylene tubing was introduced into the superficial renal cortex at the time of operation. The needle was passed along under the kidney capsule, about 2 mm below the surface, and remained *in situ* throughout the balance of the experiment. During the occlusion period, one minute prior to starting the flow, 50 to 100 μc of $K^{42}Cl$ contained in not more than 0.1 ml of water was injected through the catheter into the renal parenchyma. The stop flow specimens were subsequently counted in a well-type scintillation counter. It was postulated that a portion of the injected K^{42} would diffuse into the adjacent renal tubules during the one minute period prior to starting the urine flow, and that the isotope would thus label urine contained within the contiguous renal cortex during the occlusion period. The absence of isotope in the first specimens collected indicated that these came from the medullary or inner cortical portion of the distal tubular system. As shown in Figures 3 and 4, the K^{42} appeared in a sharp peak and then diminished, presumably because of more rapid urinary transit through the area containing the isotope, and because of removal of isotope in the renal circulation.

In all experiments, inulin was infused at a constant rate for at least one hour prior to ureteral occlusion. Creatinine was injected intravenously one minute before starting flow, in order to label new glomerular filtrate (1). Specimens were analyzed for freezing point de-

TABLE I

*Changes in osmolality and inulin concentration in stop flow experiments conducted during water diuresis**

Dog	Weight	Sustaining infusion		Free urine flow			Stop flow maximum values		Plasma osmolality
		Rate	Composition	Rate	U/P inulin	U_{os}	U/P inulin	U_{os}	
	kg	*ml/min*	*mEq/L*	*ml/min*					
B	16.3	4.0	30 NaCl 10 KCl	3.95	11.8	39	114	234	299
I	9.7	3.7	60 NaCl 10 KCl	2.52	15.0	153	126	419	267
R	13.7	7.3	30 NaCl 10 KCl	5.54	7.5	52	111	286	263

* Each dog received an initial intravenous water load (2.5 per cent glucose in water) of 3.0 to 3.7 per cent of body weight.

TABLE II

*Changes in urine osmolality during ureteral occlusion in water loaded dogs infused with isotonic electrolyte solutions**

Dog	Sustaining infusion		Free urine flow		Plasma osmolality	Stop flow Maximum U_{os}
	Rate	Composition	Rate	U_{os}		
	ml/min	*mEq/L*	*ml/min*			
D	6.0	120 NaCl 20 KCl	5.0	150	285	328
E	6.0	110 NaCl 30 KCl	4.15	165	282	414
F	6.0	80 NaCl 60 KCl	4.70	202	273	579

* Each dog received an initial intravenous water load (2.5 per cent glucose in water) of 3.9 to 4.1 per cent of body weight.

pression, and for concentrations of inulin, creatinine, sodium, potassium, and chloride. Plasma electrolytes, osmolality, and inulin concentration were also determined. Blood was withdrawn just prior to or immediately after the occlusion period. For the sake of uniformity a stop flow period of eight minutes was utilized in all experiments reported herein. Two control (free flow) clearance periods were collected immediately before and shortly after each stop flow period.

Two groups of dogs were given initial intravenous water loads, and subsequently infused with either hypotonic or isotonic electrolyte solutions. The infusion data from these studies are detailed in Tables I and II. The procedure on animals given hypertonic sodium chloride solution is summarized in Table III. Two dogs were given sodium sulfate, 120 mmoles per L, and potassium chloride, 40 mEq per L, at rates of 6.0 ml per minute. Each also received acetazolamide; a 100 mg priming dose, one hour before stopping flow, and a sustaining dose of 10 mg per kg of body weight per hour.

Urine and plasma osmolality was determined by the method of Bowman, Trantham and Caulfield (9). A special adapter was used for determinations of the 0.17 ml samples. Inulin, creatinine, and electrolytes were measured by methods previously outlined for this laboratory (10).

RESULTS

I. Osmolality and inulin concentrations

A. Water diuresis. Figure 2 depicts the results of an eight minute period of stop flow performed during a brisk water diuresis. In the first specimen collected, representing the most distal portion of the tubular system, osmolality and the urine to plasma concentration ratio for inulin (hereafter designated as U/P inulin) are markedly increased above the corresponding free flow (control) levels. Both values decrease sharply in specimens from less distal segments of the tubule. The concentration curves, plotted on a proportional scale, diminish in a parallel fashion in specimens derived from the distal tubular segments. An essentially identical pattern has been observed in two other dogs similarly water loaded and infused with hypotonic electrolyte solutions. Maximal osmolality and U/P inulin always appeared in specimens from the most distal tubular segment. These data are summarized in Table I.

TABLE III

Changes in osmolality and inulin concentration in stop flow experiments conducted during sodium chloride diuresis

Dog	Weight	Infusion* rate	Free urine flow			Plasma osmolality	Stop flow maximum values		Site of maximal U/P inulin
			Rate	U/P inulin	U_{os}		U/P inulin	U_{os}	
	kg	*ml/min*	*ml/min*						%
C	14.3	6.0	3.26	14.4	346	300	118	548	32
G	10.7	4.0	3.40	7.4	256	330	89	463	28
M	11.9	6.0	5.25	5.4	314	301	89	507	38

* Infusion of sodium chloride, 200 mEq per L.

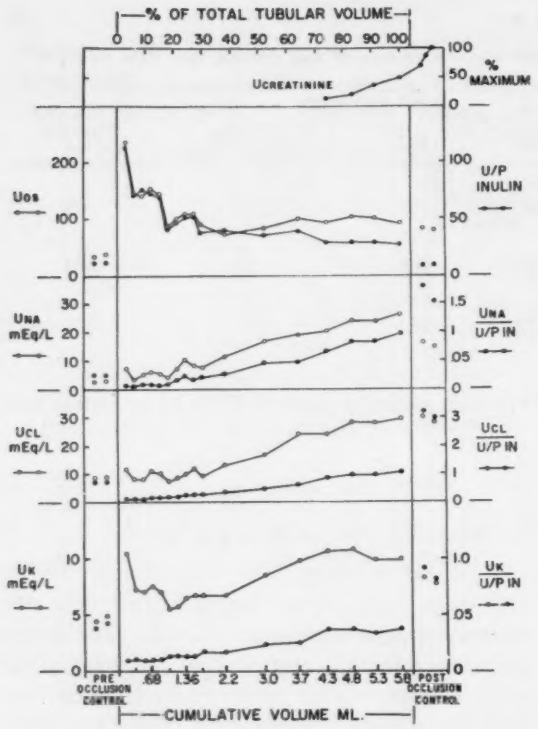

FIG. 2. STOP FLOW STUDY CONDUCTED DURING WATER DIURESIS (DOG B). In this and subsequent figures, the most proximal stop flow specimen is arbitrarily designated as that which contained approximately 50 per cent of the peak concentration of creatinine (2). Subsequent specimens represent predominantly new glomerular filtrate, and are not plotted. The total cumulative volume, plotted along the lower abscissa, includes the most proximal specimen, according to the above designation. For the sake of comparison between studies, the cumulative volume is divided into percentages of total volume, and plotted along the upper abscissa.

Maximal osmolality achieved during stop flow varied, but appeared to be directly related to the previous control urine osmolality. A similar relationship was observed in three other dogs which were initially water loaded and subsequently infused with isotonic electrolyte solutions (Table II), and in dogs infused with hypertonic sodium chloride solution (Table III).

B. Solute diuresis. Three dogs were infused with hypertonic sodium chloride solution (200 mEq per L) and stop flow performed when a solute diuresis had been established. A representative experiment is shown in Figure 3, and the data from all experiments are summarized in Table III. In each case urine osmolality was maximal in the most distal specimen. Only a gradual fall in osmolality occurred in specimens from the more proximal segments of the tubule. The pattern of U/P inulin concentration differed strikingly from that observed during water diuresis. A maximum concentration does not occur in the most distal specimen. There is instead a marked concentration peak in specimens from a more proximal portion of the tubular system (Figure 3). This has been a constant finding in all dogs infused with hypertonic sodium chloride solution, and presumably secreting endogenous vasopressin. As shown in Table III, the U/P inulin peak appears when between 28 and 38 per cent of the total fluid, up to the appearance of new glomerular filtrate, has been collected. A less striking and more diffuse peak was observed following ureteral occlusion performed during infusion of 120 mmoles per L Na_2SO_4 (Figure 4), and 2 per cent mannitol with 200 mEq per L NaCl. This peak was virtually absent during hypertonic mannitol diuresis (Figure 5).

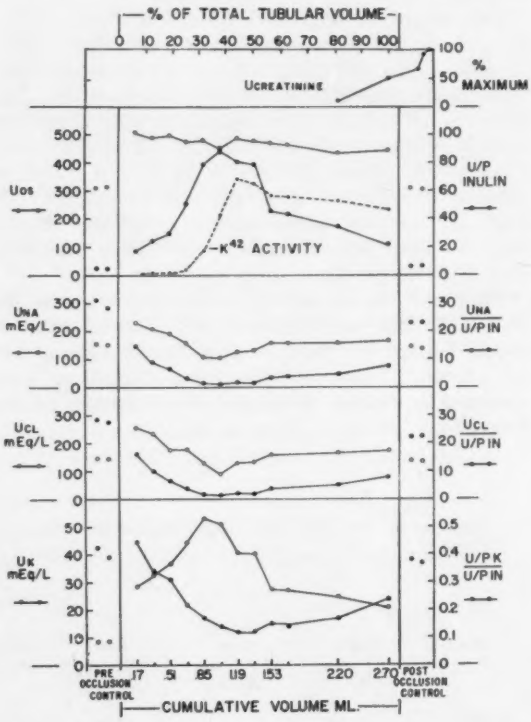

FIG. 3. STOP FLOW STUDY CONDUCTED DURING HYPERTONIC SODIUM CHLORIDE INFUSION (DOG M). Infusion: 200 mEq per L of NaCl; rate = 6.0 ml per minute. K^{42} plotted as counts per minute on a relative scale.

Further localization of the site of concentration of inulin is provided by relating this area to the concentration patterns for the various electrolytes during stopped flow. In each experiment maximum inulin concentration occurred at the site of the lowest chloride concentration, when the latter was divided by U/P inulin to correct for changes in water content within the tubular fluid. That this relationship is not solely due to the high U/P inulin in those specimens is indicated by the fact that the lowest absolute concentrations of sodium and chloride occurred in the same or adjacent specimens. It is therefore apparent that during stop flow considerable reabsorption of sodium chloride occurs in an area identical to or in close proximity to the site of maximal concentration of inulin. Another method for the localization of these effects has been the intracortical injection of $K^{42}Cl$ during stop flow. This has been done during sodium chloride and sodium sulfate diuresis. In each case (Figures 3 and 4) the appearance of high K^{42} activity coincided

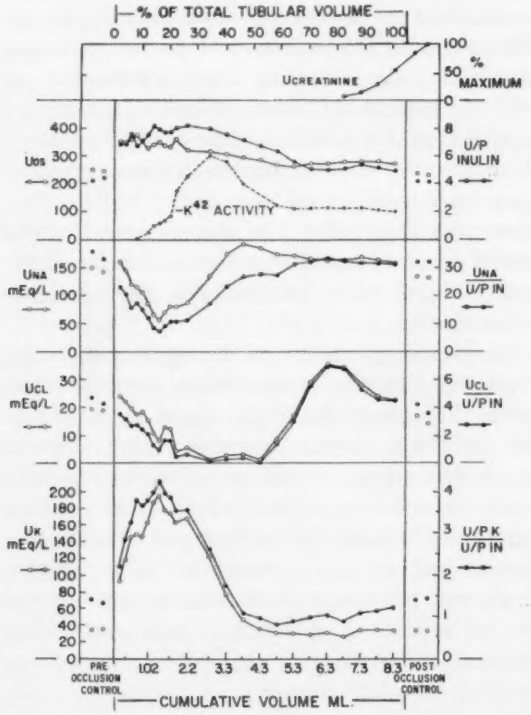

FIG. 4. STOP FLOW STUDY CONDUCTED DURING SODIUM SULFATE DIURESIS (DOG P). Infusion: 120 mmoles per L Na_2SO_4, 40 mEq per L of KCl, plus acetazolamide; rate = 6.0 ml per minute.

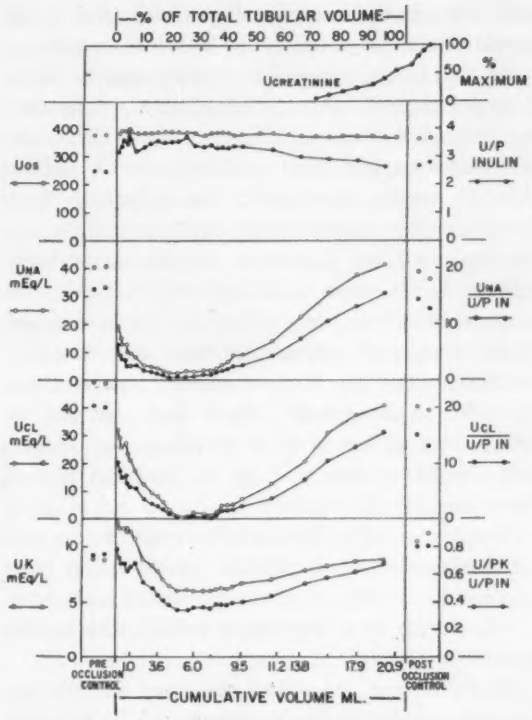

FIG. 5. STOP FLOW STUDY CONDUCTED DURING MANNITOL DIURESIS (DOG K). Infusion: 15 per cent mannitol plus 30 mEq per L of NaCl; rate = 10 ml per minute.

with the area of maximum inulin concentration. This suggests that the site of concentration of inulin was in that segment of the distal nephron contained within the renal cortex, the distal convoluted tubule.

II. Concentration patterns of electrolytes during stop flow

A. Sodium and chloride. The concentration patterns for sodium and chloride shown in Figure 3 are characteristic of those observed during stop flow performed during sodium chloride diuresis. As has been noted, minimal concentrations are observed in or closely adjacent to those samples in which U/P inulin and K^{42} activity are at a maximum. More distal specimens contain a higher concentration of these ions. A similar pattern of distal sodium chloride concentrations is present in dogs infused with hypertonic mannitol (Figure 5).

During stop flow, the sodium and chloride concentrations, corrected for inulin, are consider-

ably lower in the most distal specimen than in the previous control urine. The absolute concentrations, that is, uncorrected for inulin, may be lower than the control values in experiments done during mannitol diuresis (Figure 5). These observations suggest that reabsorption of sodium chloride occurs throughout the collecting duct. However, this concentration difference might also be explained by expansion of the intratubular volume in the most distal segment by fluid from a more proximal area, which had been exposed to the region of sodium transport and therefore contained very low concentrations of sodium and chloride. Consequently, these data can not be interpreted as unequivocal evidence for sodium and chloride reabsorption in the terminal part of the collecting duct system.

Because of difficulties in interpretation, the patterns of sodium and chloride concentration from specimens derived from the proximal convoluted tubule during stop flow are not dealt with in this report.

B. Potassium. A distal secretory site for potassium can be readily demonstrated by the stop flow technique. To facilitate the attainment of U/P potassium over U/P inulin ratios greater than 1, the sodium salt of a nonreabsorbable anion has been infused. Figure 4 shows the result of a study in which 120 mmoles per L of Na_2SO_4 and 40 mEq per L of KCl, as well as acetazolamide, were infused. The U/P potassium concentration ratio, corrected for inulin, rose to a value exceeding 4.0 in the distal tubule, considerably above the free flow ratio of 1.3. K^{42} was injected into the renal cortex during the period of ureteral occlusion. The corrected potassium concentration exceeded free flow values in all specimens from the distal 40 per cent of the tubular system, including those in which K^{42} activity was maximal. As shown in Figure 4, the maximum potassium concentration appeared distal to the isotope peak. The data suggest that secretion of potassium occurred in the distal convoluted tubule and at a more distal site, in the collecting ducts.

In both experiments in which sodium sulfate was infused, that specimen containing the highest U/P potassium over U/P inulin ratio also contained the highest absolute potassium concentration. As shown in Figure 3, a maximum potassium concentration is observed during sodium chloride diuresis which corresponds closely in location to that occurring during sulfate infusion. Whereas correction for inulin concentration obliterates this peak observed during sodium chloride diuresis, it is probable that this correction, as applied to potassium, is misleading. As the fluid in that segment of the tubule in which potassium secretion occurs remains in prolonged contact with the epithelium, during stop flow, it is probable that some maximal intraluminal potassium concentration is achieved. If so, the absolute potassium concentration, at the site of secretion, is independent of the movement of water out of the tubule, and thus of the intraluminal inulin concentration. Consequently, the absolute potassium concentration in distal tubular specimens may well be more representative of events occurring within the tubular lumen during the period of ureteral occlusion than are the "corrected" values.

As shown in Figures 3 and 4, the potassium concentrations in specimens from the most distal portion of the tubule are considerably higher than those observed in the previous control periods. This suggests that secretion of potassium occurs throughout the collecting duct system, but, as with the question of sodium chloride reabsorption in that area, the possibility that reabsorbed water from the most distal specimens has been replaced by potassium-rich fluid from higher in the tubule cannot be excluded. The data suggest that the site of maximal secretion is proximal to the terminal collecting ducts, and includes the distal convoluted tubule.

In stop flow performed during mannitol diuresis, the potassium concentration remains at low levels throughout the distal tubular system (Figure 5), and no peak comparable to that observed in sodium sulfate or sodium chloride diuresis is seen. The lowest concentrations occur in those specimens in which the sodium and chloride concentrations are at a minimum. In contrast, maximum potassium concentrations are observed in the same or in a closely adjacent area when ureteral occlusion is carried out during sodium chloride or sulfate diuresis.

DISCUSSION

As stated previously, the technical shortcomings of the stop flow method preclude precise lo-

calization of certain tubular functions. During experiments performed during water or sodium chloride diuresis, it is expected that reabsorption of water in the distal tubular segments will result in a movement of the intratubular fluid column in a distal direction during the period of stop flow. Consequently various concentration patterns are best evaluated in relation to one another rather than in relation to their absolute location among the serial specimens collected after release of occlusion. An attempt at absolute location of functions occurring in the distal convoluted tubule has been made by the use of K^{42} labeling, however. Some movement of labeled fluid from the distal convolution toward a more distal site might be anticipated because of continued water reabsorption in the latter area. It has been observed, however, that the initial isotope peak appears at essentially the same point in water diuresis (38 per cent of the total tubular volume) as in sodium chloride or sulfate diuresis, despite the fact that relatively more water reabsorption occurs in the terminal collecting ducts during water diuresis, as evidenced by the marked increment in inulin concentration in the initial stop flow specimens. This suggests that the short time interval between injection of the isotope and starting urine flow minimizes migration of the labeled tubular fluid.

It is generally agreed that vasopressin increases water permeability in the distal convoluted tubule where osmotic equilibration of hypotonic precursor fluid with isotonic interstitial fluid and plasma results (11). Presumably vasopressin has a similar effect in the collecting ducts, where concentration of the urine is achieved by diffusion of water along an osmotic gradient created by the transport of sodium into the medullary interstitial fluid. The present data are fully compatible with the view that vasopressin has such an action in the distal convoluted tubule. The marked rise in inulin concentration during the period of ureteral occlusion (Figure 3) denotes a considerable loss of water from the tubule lumen, and the data suggest that this has occurred in the region of the distal convolution. In contrast to this effect observed during hypertonic sodium chloride infusion, no similar peak in inulin concentration occurs during water diuresis (Figure 2). In fact, the absence of a detectable increase in U/P inulin in the area of the distal convolution suggests a high resistance to net water movement in that area when vasopressin is absent.

During ureteral occlusion in dogs undergoing sodium chloride diuresis the maximal inulin concentration attained is 8 to 16 times greater than the free flow value. This increment can not be solely accounted for by osmotic equilibration of hypotonic precursor fluid with an isotonic interstitium (11, 12). The observation that sodium was reabsorbed in the same segment of the tubule appears to explain the marked increase in inulin concentration in samples from that area. Water diffuses out of the tubules, in the presence of vasopressin, as sodium chloride is transported out of the tubular fluid. A process comparable to that occurring in the proximal tubule is envisioned except that in the distal tubule the capacity for outward transport of sodium (or a restricted back diffusion) allows for more complete removal of sodium from the tubular fluid.

The association, apparently in the region of the distal convoluted tubule, of vasopressin-sensitive water permeability and a mechanism capable of transporting sodium (chloride) against a high electrochemical gradient has important implications concerning the ability of the kidney to elaborate a highly concentrated urine. These processes, acting in conjunction, will result in a considerable reduction in the volume of fluid delivered to the collecting ducts. The water reabsorbed in the process is readily removed by the vascular supply of the renal cortex. On the other hand, water diffusing from the collecting ducts will dilute the medullary interstitial fluid, and tend to dissipate the osmotic gradient created by the active transport of sodium into this area. Therefore, at any constant degree of water permeability of the collecting ducts, and constant rate of sodium transport into the medulla and blood flow through the capillary loops, the final osmolality of the urine will tend to vary inversely with the volume of fluid entering the collecting duct system per unit of time. Removal of water in the distal convolution also results in an increased urea concentration in the fluid entering the concentrating area. This will in turn effect an increased rate of diffusion of urea into the medullary interstitial fluid, with a resultant increase in its osmolality and that of the final urine (13).

The relationship between the volume of fluid delivered to the collecting ducts and the final urine osmolality is emphasized by the studies of Berliner and Davidson (10). Hypertonic urine was elaborated, despite the absence of vasopressin, when filtration rate was sufficiently reduced in one kidney by controlled constriction of the renal artery. It was concluded that this procedure resulted in the delivery to the collecting ducts of a small volume of dilute urine, and that sufficient equilibration with a hypertonic interstitial fluid occurred to result in the excretion of hypertonic urine. That diffusion of water across the collecting ducts may occur in the absence of vasopressin is further indicated by the present study. The marked rise in U/P inulin and osmolality observed in ureteral occlusion performed during water diuresis indicates that water diffused from collecting ducts to interstitium along an osmotic gradient. It is probable that this gradient was higher in water diuresis than in sodium chloride diuresis, because of the low osmolality of the tubular fluid in the former. As a result, the inulin concentration in the most distal specimen showed a greater increment above control levels in water diuresis (Figures 2 and 3).

During mannitol diuresis, because of the presence of nonreabsorbable solute, water movement out of the distal convoluted tubule, as well as the proximal tubule, is minimized, and a large volume of fluid enters the collecting duct system per unit of time. The rate of movement of water out of the collecting ducts into the medulla attains maximal values (14), and sodium and urea are washed out of the interstitial fluid (6, 15). The resultant decrease in osmolality of the interstitial fluid accounts for the limited osmolar U/P ratio attainable during mannitol diuresis and for the failure to achieve a significant increase in osmolality during ureteral occlusion. It has recently been proposed (6), however, that the latter observations can be accounted for by a decreased efficiency of the medullary countercurrent multiplier mechanism, resulting from a high rate of urine flow through the loops of Henle. According to this hypothesis, the resultant fall in osmotic gradient within the renal medulla leads to diminished water reabsorption from the collecting ducts, a conclusion which is incompatible with the high rates of solute-free water abstraction generally observed during mannitol diuresis.

The stop flow technique does not appear to demarcate processes occurring in the loops of Henle. Micropuncture studies have demonstrated that fluid entering the distal convolution is hypotonic to plasma, regardless of the final concentration of the urine (11, 12). No hypotonic samples appear in occlusion studies performed during NaCl diuresis, however, presumably because the fluid must subsequently pass through the distal convolution and the collecting ducts, where concentration occurs. The present technique does not distinguish a distinct area of sodium transport corresponding to that thought to be present in the ascending limb of Henle's loop (11). It instead suggests that this area is directly contiguous with the sodium transport mechanism present in the distal convoluted tubule.

The present study is compatible with sodium chloride reabsorption in the collecting ducts, in accord with the observations of Hilger, Klümper and Ullrich, obtained by microcatheterization of the collecting ducts of the hamster (16). Utilizing two catheters, fluid samples were withdrawn from two different sites at accurately determined distances from the tip of the papilla. Concentration differences between the proximal and distal samples were interpreted as representing alterations in the tubular fluid imposed by diffusion or active transport across the collecting duct epithelium. In repeated observations, the more distal specimen contained a higher inulin concentration and a lower sodium concentration than the sample obtained at a more proximal site. This was interpreted as evidence of active transport of sodium, although whether this was exclusively by means of cation exchange or transport in association with an anion, was not established. The possibility that sodium chloride transported from the collecting ducts contributes to the establishment of the osmotic gradient necessary for the concentration of the urine has therefore been considered. Outward transport of sodium chloride will dilute the remaining tubular fluid unless it is accompanied by water, in which case no change in osmolality will occur. It is possible that sodium transported from the collecting ducts contributes to the total sodium content of the medulla, but

only that part of the interstitial sodium transported from some portion of the tubule which is relatively impermeable to water (such as the ascending limb of the loop of Henle) can be responsible for the establishment of the high sodium concentration necessary for the concentrating process. Sodium transport from the collecting duct may effect water removal from the tubular lumen, by the same process postulated for the distal convolution, and thus result in the delivery of a smaller volume of urine to the distal portion of the collecting duct system. Analyses of slices from concentrating kidneys (15, 17) indicate that urea reaches ever higher concentrations as the tip of the papilla is approached, indicating that urinary concentration proceeds in a progressive fashion down the medulla and that the maximum concentration is achieved at the terminal end of the duct system. Consequently, any process which progessively reduces the volume of tubular fluid during its transit down the collecting duct will enhance the degree of hyperosmolality attained in the final urine.

Previous studies, using the stop flow technique, have indicated a distal secretory site for potassium (2, 18). The present data are in accord with these observations. It has been suggested that a reabsorptive mechanism for potassium also exists in the distal nephron (18–20). This conclusion is based on the observation that, during mannitol diuresis, low potassium concentrations are found in specimens from the distal system. Similar observations during mannitol diuresis have been made in this laboratory. This finding presents an apparent paradox, since a secretory site can apparently be demonstrated in the same or in a closely contiguous area under other conditions. It is worthy of note, however, that the low potassium concentrations noted during mannitol diuresis are present in the same specimens which contain the lowest concentrations of sodium and chloride, and that the latter ions are virtually completely removed from distal urine in ureteral occlusion performed during mannitol diuresis. This association suggests that, if, as considerable evidence suggests (21, 22), potassium secretion occurs as a cation exchange process, the virtual absence of an exchangeable cation (Na) precludes appreciable potassium secretion into this urine specimen as it subsequently flows past the potassium secretory site. On the other hand, when NaCl or Na_2SO_4 is infused, the concentration of sodium in the tubular fluid never attains extremely low levels. Thus, in NaCl diuresis a concentration peak for potassium can be demonstrated in proximity to, and perhaps slightly distal to, the distal site of maximal NaCl transport. In Na_2SO_4 diuresis, secretion unequivocally occurs in the same area. It appears most likely then, that rather than demonstrating a distal reabsorptive site for potassium, ureteral occlusion during mannitol diuresis produces a change in the tubular fluid which prevents subsequent potassium secretion into a segment of the trapped fluid.

SUMMARY

1. Utilization of a modified technique for stop flow analysis has permitted study of distal renal tubular functions under a variety of experimental conditions, including water, sodium chloride, sodium sulfate, and mannitol diuresis.

2. In accord with previous studies, the data suggest that the epithelium of the distal convolution is highly permeable to water in the presence of vasopressin. In contrast, during water diuresis, a considerable restriction to the diffusion of water is exhibited in the same area. It has been shown that water diffuses out of the collecting ducts in the absence of vasopressin, as has been concluded by previous workers.

3. Sodium chloride reabsorption occurs in the region of the [illegible]al convoluted tubule. Under certain conditions this process may occur against a high electrochemical gradient. It has been demonstrated that vasopressin-dependent water permeability and sodium chloride reabsorption occur coextensively, apparently in the cortical area of the distal tubular system. This association results in an effective reduction in the volume of fluid delivered to the concentrating area, and consequently enhances the ability of the kidney to elaborate a highly concentrated urine.

4. Potassium secretion occurs in the distal tubular system during ureteral occlusion. It is suggested that previous conclusions as to the presence of a potassium reabsorptive mechanism in the distal tubule are erroneous, since the data were obtained under conditions in which the sodium con-

centration of the tubular fluid was extremely low, thereby imposing a limit on subsequent cation exchange, potassium for sodium.

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BILATERAL STUDIES OF CEREBRAL OXYGEN UPTAKE IN YOUNG AND AGED NORMAL SUBJECTS AND IN PATIENTS WITH ORGANIC DEMENTIA

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A relation between reduced cerebral oxygen uptake ($CMRO_2$), measured by the inert gas method of Kety and Schmidt (1), and impaired mental function has been described in many clinical conditions. In patients with chronic degenerative cerebral diseases, such a relationship was first noted by Freyhan, Woodford and Kety in their study of aged psychotic patients (2). This finding has since been confirmed by many investigators studying related clinical problems (3–7). In general, a reduction of $CMRO_2$ was found to be associated with a proportionate reduction of the cerebral blood flow (CBF).

These studies showed considerable overlapping between the values obtained in the deteriorated patients with organic brain disease and in the normal control patients. Recently, Lassen, Munck and Tottey reported a study of CBF and $CMRO_2$ in middle-aged patients with mental impairment due to diffuse, degenerative brain diseases (8). Compared to normal control subjects of similar age, *all* patients with organic dementia had subnormal $CMRO_2$, while the CBF showed some degree of overlapping. Furthermore, the degree of reduction of $CMRO_2$ corresponded roughly with the degree of mental deterioration. Psychological tests were employed to assess mental abilities in this study, while the krypton^{85} method was used to measure CBF and $CMRO_2$ bilaterally, employing simultaneous sampling from the right and left internal jugular veins. These relatively minor variations of Kety and Schmidt's method (1) were found to be of value, as they appeared to increase the accuracy of the individual observations.

The demonstration of a close correlation between mental and metabolic deterioration in cerebral function is of importance for the understanding of the pathophysiology of organic dementia. Since only 19 subjects were studied by Lassen and associates (8) it was considered desirable to collect more data of this kind. The present report is concerned with clinical and cerebral metabolic data from 25 subjects. The technique and the overall design of the study were similar to that used by Lassen and co-workers. It involved the independent physiological and psychological assessment of the subjects, data being compared only after completion of the entire study.

METHODS

Cerebral blood flow and oxygen uptake. The inert gas method of Kety and Schmidt (1) was used with the modification described by Lassen and Munck (9). This modification employs a radioactive isotope of krypton (Kr^{85}) as the inert gas instead of nitrous oxide.

Each study had a duration of about 16 minutes during which time the subject breathed atmospheric air containing 0.1 μc Kr^{85} per ml. A closed circuit system of 80 L was used. Blood samples were drawn from indwelling needles in the femoral artery and in both internal jugular veins. Twelve samples of 3 cc each were drawn anaerobically from each source for Kr^{85} analysis (Figure 1). These 36 samples and a blank sample drawn before Kr^{85} inhalation were transferred into mica-walled cuvets and the radioactivity measured automatically by 4 Geiger-Müller tubes (9). In order to avoid errors due to variations in erythrocyte sedimentation in the cuvets, the analyses were performed on hemolyzed blood; triton X-100 was used as the hemolytic agent (10). After about 4 minutes of Kr^{85} inhalation, samples were drawn for manometric O_2 and CO_2 analyses (11), pH, and spectrophotometric O_2 saturation determinations (10). The arterial hematocrit was also measured and was used to calculate the partition coefficient for Kr^{85} (9).

The calculation of the unilateral (right or left side) values of the cerebral blood flow (CBF) was carried out according to the principle of Kety and Schmidt (1) as modified by Lassen and Munck (9). Extrapolation to infinity was used, but no correction was made for the

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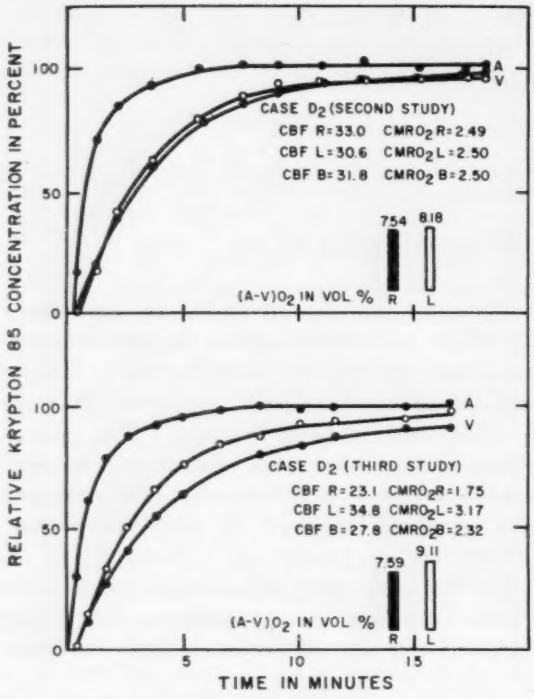

FIG. 1. TWO STUDIES IN THE SAME SUBJECT. An interval of several weeks separated the studies. The open circles represent the venous curves for the left side.

mean circulation time of the brain (9). Though theoretically sound, this correction cannot be made accurately, and it was thought preferable not to use it in the present study. For this reason the infinity values of CBF and $CMRO_2$ reported in this paper *are about 10 per cent lower* than those reported by Lassen, Munck and Tottey (8). The unilateral CBF values also were calculated to an experimental duration of 10 minutes. These values can be compared directly to those obtained by the original nitrous oxide method. The bilateral values were calculated with the assumption that both internal jugular veins carry the same volume of blood. Therefore, the means of the two venous krypton curves and the means of the oxygen contents were used in these calculations.

Clinical data and psychological methods. Twenty-five white male subjects were studied: 11 young normal subjects (Y) of average age 24 years; 5 old normal subjects (O) of average age 72 years; and 9 aged, mentally deteriorated subjects (D) of average age 74 years. The young normal subjects were medical students. They were not examined clinically as it was assumed that none suffered from chronic brain disease of a degree sufficient to impair intelligence.

The two groups of aged subjects were selected and evaluated by a psychiatrist (I.F.) who was not made aware of the circulatory and metabolic data until completion of the entire study. The grouping of these subjects as *normal* or *deteriorated* mentally was made on the basis of the overall clinical impression: the results obtained by psychometric tests were evaluated in relation to past history, emotional status (cooperativeness) and possible neurological defects. The aged subjects were selected so that the normal aged group was mentally well preserved and alert, whereas the deteriorated subjects all manifested behavioral changes which clearly suggested chronic brain derangement (dementia), with signs of "senility" and confusion (see below).

The examination included: *a*) psychiatric interview; *b*) Wechsler's Adult Intelligence Scale (WAIS) (12); *c*) Raven's Progressive Matrices (13) (including adult and children's forms); *d*) Bender Motor Gestalt (14); and *e*) a digit memory test designed to reveal fluctuations in performance (described in Reference 8). Some clinical data are given in Table I and test scores are listed in Table II. It will be noted that regarding school training and life occupation, no apparent difference existed between the normal and the deteriorated aged subjects, an impression confirmed by the more detailed interviews.

Three of the five mentally normal aged subjects were paid volunteers (O_1, O_2 and O_4), and two were patients from the Geriatrics Ward of Saint Elizabeth's Hospital, Washington, D. C. (O_3 and O_5). These two patients were hospitalized primarily because they had no one to care for them in the community. One of them (O_3) had physical infirmity, whereas the other (O_5) was admitted because of mental depression and excessive use of alcohol following his wife's death. Shortly after this patient's participation in the present study he was discharged from the hospital. The clinical impression of well preserved mental function in these five subjects was confirmed during the detailed interviews and by the results of the psychometric tests (Tables I and II).

The nine mentally deteriorated aged subjects were patients hospitalized on the Geriatrics Wards of Saint Elizabeths Hospital. Patients were sought whose hospital diagnosis was chronic brain syndrome and who manifested the characteristic symptoms of this illness, in the absence of focal neurological signs. A loss of memory more marked for recent than remote events was considered the distinguishing feature of organic brain disease. Emotional lability, defects in orientation, in comprehension and in judgment frequently accompanied the memory impairment. In addition, an attempt was made to exclude patients with a history of or with present evidence of such stigmata of functional mental illness as delusions, hallucinations, ideas of reference, and depersonalization. The endeavor to meet these ideal criteria in a hospital devoted primarily to the care of committed patients was not entirely successful. Thus one subject (D_2) had a past history of auditory hallucinations and was considered to show minimal signs of residual schizophrenia in addition to organic deficit. Two others (D_1 and D_5) had vague unsystematized paranoid notions regarding their families; however, both had ade-

TABLE I

*Descriptive data for populations tested**

	Age	Duration of hospitalization	Scholastic achievement	Occupation
	yrs	*yrs*		
Young normal subjects		None	College graduates	Medical students
N = 11	22–29			
Mean = 24.2				
Old normal subjects				
O_1	75	None	College graduate	Marine insurance (U. S. Govt.)
O_2	66	None	College graduate	Civil engineer
O_3	79	3	Tenth grade	Florist clerk
O_4	72	None	Eighth grade	Engraver (U. S. Govt.)
O_5	69	1	1 Year college	Telegraph operator
Mean = 72.2				
Subjects with organic dementia (chronic brain syndrome)				
D_1	67	1	High school graduate	Commander, U.S.N.
D_2	69	2	College graduate (LL.B.)	Lawyer
D_3	77	2	High school graduate	Unknown
D_4	74	1	First grade	Laborer (U. S. Govt.)
D_5	73	2	Ninth grade	Insurance salesman
D_6	72	1	High school graduate	Engraver (U. S. Govt.)
D_7	75	2	Sixth grade	Post Office clerk
D_8	84	3	High school graduate	Clerk (U. S. Govt.)
D_9	77	1	High school graduate	Clerk (U. S. Govt.)
Mean = 74.2				

* Positive findings on neurological examination: D_4 had moderate Parkinsonism and left homonymous visual field defect; D_7 had moderate Parkinsonism; and D_9 had aphasia and apraxia, but no other neurological symptoms or signs.

quate histories of work and family adjustment. Finally, two additional patients presented focal neurological signs (Tables I and II). Although none was capable of managing his affairs outside the hospital, three patients had ground parole privileges. The remaining six subjects were so confused as to require assistance in the simple tasks of everyday life; they were unable to find their way about, to dress themselves, and were usually disoriented in all spheres. Two of the deteriorated subjects, D_8 and D_9, were considered to be more severely affected than the others. These two subjects were deliberately chosen in order to include in the study some subjects with an extreme degree of impairment. None of the patients was a behavior problem and all except

TABLE II

Scores on psychometric tests

	WAIS subtests (standard scores)										
	Information	Comprehension	Arithmetic	Similarities	Digit span	Vocabulary	Digit symbol	Picture complet.	Block design	Picture arrang.	Raven's matrices*
O_1	17	15	11	16	7	17	7	12	8	7	49
O_2	19	18	17	1	12	18	8	13	12	13	64
O_3	9	10	10	7	7	10	5	9	8	6	32
O_4	14	19	13	11	9	15	8	12	12	7	46
O_5	16	17	15	11	12	19	7	11	10	6	59
D_1	17	18	15	15	14	14	6	14	6	6	38
D_2	14	14	13	10	14	17	7	9	7	7	41
D_3	13	10	9	12	14	19	NT†	NT	9	NT	NT
D_4	5	7	5	4	6	2	0	5	0	1	0
D_5	5	8	10	4	9	11	7	6	9	6	39
D_6	9	9	15	8	11	11	3	7	5	5	34
D_7	9	6	8	2	1	10	NT	7	5	0	24
D_8	7	8	4	5	6	9	NT	NT	NT	NT	NT
D_9	Untestable on all tasks										

* Total items correct on combined children's and adult forms.
† Not tested.

one, D_9, were capable of performing at least some psychological tests.

The etiology of the chronic brain syndrome in these patients was obscure. No subject had a history of syphilis, apoplexy, severe cerebral anoxia, trauma, or any of the other usual causes of diffuse cerebral disease. Only two patients presented findings suggestive of cerebral vascular insufficiency, and none suffered from severe arterial hypertension. These facts and the insidious, gradually progressive nature of the mental derangement would seem to argue against a general presumption of vascular ("arteriosclerotic") etiology for cognitive changes observed. The role of cerebral vascular insufficiency cannot, however, be entirely excluded on this basis.

RESULTS

CBF and $CMRO_2$ in the various clinical groups. Table III lists the unilateral and bilateral CBF and $CMRO_2$ values for each subject, and the means of these values in the three clinical groups.

TABLE III

Bilateral cerebral blood flow and oxygen uptake in 25 subjects

		10 Minute values*						Infinity values*					
		CBF			$CMRO_2$			CBF			$CMRO_2$		
Subjects	Age	R	L	Bil.	R	L	Bil.	R	L	Bil.	R	L	Bil.
	yrs												
I. Young normal subjects													
Y_1	28	62.4	63.3	62.8	5.45	5.20	5.33	56.8	54.9	55.8	4.96	4.51	4.73
Y_2	29	42.2	45.0	43.6	3.83	4.07	3.95	34.5	39.4	36.8	3.13	3.57	3.33
Y_3	24	52.7	65.6	58.4	3.37	4.09	3.69	44.4	57.3	50.0	2.84	3.58	3.16
Y_4	22	42.9	48.2	45.4	3.35	3.80	3.56	36.8	42.3	39.4	2.88	3.33	3.09
Y_5	23	47.7	58.2	52.4	3.34	4.10	3.68	39.5	49.7	44.0	2.77	3.50	3.09
Y_6	22	49.7	46.5	48.0	3.65	3.06	3.34	43.9	42.8	43.3	3.23	2.81	3.01
Y_7	25	50.3	52.2	51.2	3.75	3.64	3.70	42.2	40.8	41.5	3.15	2.84	3.00
Y_8	28	49.0	52.2	50.5	3.35	3.13	3.24	44.0	46.8	45.4	3.01	2.81	2.91
Y_9	24	54.5	61.9	58.0	3.47	3.63	3.55	44.8	47.3	46.0	2.85	2.78	2.82
Y_{10}	21	42.5	52.3	46.9	2.72	3.53	3.08	38.3	45.1	41.4	2.45	3.04	2.72
Y_{11}	24	49.4	48.8	49.1	3.26	2.92	3.09	42.0	41.5	41.7	2.77	2.49	2.62
Mean†	24.2	48.09	53.09	50.35	3.409	3.597	3.488	41.04	45.30	42.95	2.908	3.075	2.975
SD		4.29	6.78	4.91	0.310	0.439	0.289	3.58	5.32	3.69	0.231	0.390	0.213
II. Old normal subjects													
O_1	75	37.2	39.0	38.1	3.57	3.66	3.62	31.7	29.1	30.3	3.04	2.73	2.88
O_2	66	45.2	53.7	49.1	2.94	3.87	3.36	38.0	42.8	40.3	2.47	3.08	2.76
O_3	79	42.2	45.6	43.8	3.15	3.34	3.24	35.2	38.9	37.0	2.63	2.85	2.73
O_4	72	62.2	72.1	66.8	2.84	3.12	2.97	55.8	65.2	60.1	2.54	2.82	2.67
O_5	69	59.1	48.0	53.0	3.32	2.60	2.93	53.1	40.6	46.0	2.98	2.20	2.54
Mean	72.2	49.18	51.68	50.16	3.164	3.318	3.224	42.76	43.32	42.74	2.732	2.736	2.716†
SD		10.91	12.57	10.86	0.293	0.494	0.286	10.94	13.30	11.24	0.261	0.326	0.125
III. Subjects with organic dementia (chronic brain syndrome)													
D_1	67	50.7	51.4	51.0	3.09	2.89	2.99	41.1	45.5	43.2	2.51	2.56	2.55
D_2	69	34.0	39.2	36.4	2.87	3.57	3.19	26.6	30.6	28.5	2.25	2.78	2.50
D_3	77	34.8	31.5	33.1	3.00	2.43	2.71	32.3	29.1	30.6	2.78	2.25	2.50
D_4	74	33.8	32.5	33.1	3.19	2.54	2.85	30.6	27.1	28.7	2.89	2.12	2.47
D_5	73	39.0	34.6	36.7	3.01	2.56	2.77	33.3	32.0	32.6	2.57	2.37	2.46
D_6	72	58.7	44.0	50.3	3.45	2.49	2.90	51.1	35.3	41.8	3.00	1.99	2.41
D_7	75	41.0	36.1	38.4	3.08	2.65	2.85	33.3	28.9	30.9	2.50	2.12	2.30
D_8	84	42.8	36.6	39.5	2.63	2.08	2.34	36.7	30.9	33.6	2.26	1.76	1.99
D_9	77	32.0	25.9	28.6	2.23	1.57	1.86	23.9	18.9	21.1	1.66	1.15	1.37
Mean	74.2	40.76	36.87§	38.57	2.950	2.531§	2.718§	34.32	30.92	32.33	2.491	2.122§	2.281§
SD		8.91	7.43	7.59	0.350	0.543	0.395	8.07	7.07	6.79	0.405	0.474	0.380
IV. Repeat studies in three subjects with organic dementia													
D_2-II	68	40.0	41.6	40.8	3.02	3.40	3.21	33.0	30.6	31.8	2.49	2.50	2.50
D_2-III	68	28.5	42.9	34.2	2.16	3.91	2.86	23.1	34.8	27.8	1.75	3.17	2.32
D_5-II	73	37.3	30.9	33.8	3.14	2.30	2.68	32.5	24.6	28.0	2.74	1.83	2.22
D_7-II	73	41.2	39.7	40.4	2.39	2.58	2.48	31.0	30.5	30.7	1.79	1.98	1.89

* Values are expressed in cubic centimeters per 100 g per minute.
† Mean value of subjects Y_2 to Y_{11} (cf. the text).
‡ Significantly lower than in young normal adults ($p < 0.05$).
§ Significantly lower than in old normal subjects ($p < 0.05$).

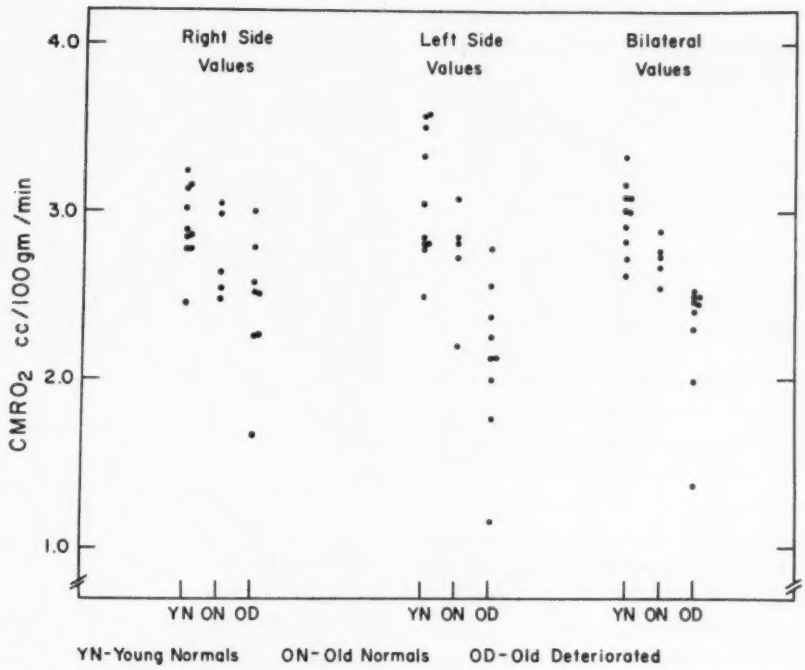

FIG. 2. SCATTERGRAM OF $CMRO_2$ VALUES IN THE VARIOUS CLINICAL GROUPS. Note that the bilateral values distinguish most sharply among the groups.

The probability levels for the pertinent differences are also indicated. These levels are based on the application of the *t* test with correction for nonhomogeneous variances (15).[1] Case Y_1 was unusual because the values for $CMRO_2$ were much higher than in any other young normal subject studied. The ratio of the sums of squares in cases $Y_2 - Y_{10}/Y_1 - Y_{11}$ was 0.127. This ratio (16) indicates that Case Y_1 differs significantly from the remainder of the cases ($p < 0.01$), and this case has therefore not been included in computation of the mean and standard deviation for the young normal group. The basis of this deviant value was not readily apparent.

Compared with young normal adults, the aged normal subjects showed a significant ($p < 0.05$) reduction of the bilateral $CMRO_2$ value calculated to infinity. In young normals a mean value of 3.0 cc per 100 g per minute was found as compared to 2.7 in aged normals. The bilateral 10 minute values of $CMRO_2$ were not significantly different at the 5 per cent level of confidence. The unilateral CBF and $CMRO_2$ values, as well as the bilateral CBF values, failed to distinguish between these two normal groups at either the 10 minute or infinity values.

The nine aged deteriorated subjects differed significantly from the aged normal controls with respect to several parameters. The bilateral and left side unilateral values for $CMRO_2$ were significantly reduced ($p < 0.05$) in the deteriorated group, whereas the $CMRO_2$ values for the right side were not significantly different. The cerebral blood flow values did not differ significantly in the two groups with the exception of the 10 minute CBF value on the left side; this was significantly reduced ($p < 0.05$) in the deteriorated subjects. It may also be noted that cases D_8 and D_9, who clinically appeared most deteriorated, also had the lowest $CMRO_2$ values.

In the present study, the bilateral $CMRO_2$ values provide a more clear-cut separation of the clinical groups than do either the right or left side values. This is clearly shown in Figure 2, in which the three sets of values are plotted for each group.

[1] We wish to thank Dr. Seymour Geisser, Biometrics Section, National Institute of Mental Health, for statistical advice.

TABLE IV

Correlations of cerebral oxygen uptake with psychometric data listed in Table II

	$CMRO_2$ (infinity values)			
	N	Right	Left	Bilateral
1. Picture arrangement	10	−0.13	0.77*	0.71*
2. Picture completion	10	0.00	0.62†	0.68†
3. Digit symbol	9	0.38	0.57	0.40
"Perception" factor‡	10	0.13	0.78*	0.74*
4. Digit span	13	0.04	0.32	0.29
5. Arithmetic	13	0.13	0.57*	0.57*
6. Block design	12	0.22	0.63†	0.55†
"Immediate memory" factor‡	13	0.01	0.58†	0.52†
7. Similarities	13	0.15	0.60†	0.64†
8. Information	13	0.04	0.61†	0.60†
9. Vocabulary	13	0.04	0.41	0.44
"Verbal" factor‡	13	0.08	0.59†	0.59†
Raven's matrices	10	0.18	0.44	0.56†

* One-tailed test, $p < 0.01$.
† One-tailed test, $p < 0.05$.
‡ Based on combining weighted scores of the three preceding subtests. For the "perception" factor, D_7 was prorated for Test 3 on the basis of Tests 1 and 2; for the "immediate memory" factor, D_8 was prorated for Test 6 on the basis of Tests 4 and 5.

CBF and $CMRO_2$ compared with psychometric data. Table IV lists correlation coefficients and their levels of significance calculated from the extrapolated $CMRO_2$ values and the psychometric data of the aged normal and the aged deteriorated group. The raw scores were correlated for individual subtests of the WAIS. The three factors isolated from the Wechsler-Bellevue Scale by Birren (17) among aged subjects were correlated by combining weighted scores of the WAIS subtests. D_9 was untestable and was not included in any computations. D_3, D_4 and D_8 did not complete every test (D_3 and D_8 because of impaired vision, and D_4 because of transient uncooperativeness). The N's for the various correlations therefore vary. It will be seen that there are high and significant correlations between $CMRO_2$ values on the left side and the psychometric data. The correlations between bilateral $CMRO_2$ and the psychometric data were of a similar value and significance. None of the correlations for the right side reaches significance and they are all close to zero. Calculations using the $CMRO_2$ values corresponding to an experimental duration of 10 minutes showed much the same results. The cerebral blood flow showed a distinctly lower degree of correlation with the psychometric data than did $CMRO_2$.

DISCUSSION

In young normal adults, the average cerebral blood flow was 50 cc per 100 g per minute, and the cerebral oxygen uptake was 3.5 cc per 100 g per minute, when calculated to 10 minutes of inert gas inhalation. These values are similar to those reported by investigators using the nitrous oxide method (9, 18–22). The extrapolated values, however, are somewhat lower with average values of 43 cc per 100 g per minute for cerebral blood flow and 3.0 cc per 100 g per minute for cerebral oxygen uptake.

As is evident from Table III, extrapolation caused a drop in calculated cerebral perfusion in all studies in normal subjects as well as in patients with organic dementia. An average decrease of about 15 to 20 per cent occurred, with subjects of lower perfusion showing the greater relative decrease. This decrease does not reflect a change in true cerebral perfusion since all studies were carried out in the steady state. Moreover, in all studies the cerebral venous oxygen saturation was measured on an additional blood sample collected at about the eleventh minute of inert gas inhalation; in no case was a marked change (decrease) noted when this value was compared to that obtained from the sample collected at about the fourth minute. Since the decrease in *calculated* cerebral perfusion value with increasing experimental duration is unrelated to changes in cerebral perfusion, it must be attributed directly to the method of study—the inert gas method.

The theory of inert gas uptake at the tissues, as reviewed by Kety (23), enables us to understand this decrease. Declining values during an experiment carried out in the steady state stem from differences of inert gas uptake rates within the organ studied. Animal studies by Kety et al. (24) indicate that the white matter of the brain has a much lower perfusion rate than does the grey matter, and consequently has a slower gas uptake rate. Therefore, the brain itself may be the source of the observed heterogeneity of Kr^{85} uptake. Extracerebral contamination of internal jugular blood would also be expected to result in heterogeneous inert gas uptake. This contamination is probably of the order of only a few per cent in the average case (25). Hence, it can-

not readily account for the 15 to 20 per cent drop in calculated perfusion rate produced by extrapolation.

Whatever the origin of the observed unevenness of tissue Kr^{85} uptake, only extrapolation can give a theoretically valid estimate of the blood flow of the tissues. The extrapolation, however, must be correct, i.e., it must comprise the saturation characteristics of all tissues not yet saturated at the end of the experiment at 16 minutes of inert gas inhalation. The procedure we employ undoubtedly corrects to some extent the erroneously high 10 minute values due to uneven perfusion and the resultant difference between the average inert gas tension of the tissues and the jugular blood. Therefore, the extrapolated values will approximate more closely the "true" blood flow. But since it is possible that not all slowly saturating tissues have been adequately represented by the present technique of extrapolation, the values derived can only approximate this ideal. Moreover, extrapolation increases the random experimental error of the method. Thus it may be said that the values determined on the basis of an experimental duration of 10 or 16 minutes are more *reliable* than extrapolated ones, even though the latter give more *valid* estimates of true blood flow. In the following discussion both 10 minute and infinity values are mentioned, no special significance being attached to either. In most cases the same conclusion may be reached when using either set of values. It is worth noticing, however, that in the group of old normal subjects the extrapolated value for cerebral oxygen uptake was significantly reduced ($p < 0.05$), while the reduction of the 10 minute value just fell short of reaching statistical significance ($0.05 > p < 0.10$). While this difference may be due to chance it may also reflect the possibility that extrapolation actually gives a better estimate of the parameter sought.

The discussion above implies that in many studies employing an experimental duration of 10 minutes, the normal rate of cerebral blood flow and oxygen uptake has been overestimated by about 15 per cent. The values in subjects with low cerebral perfusion have been overestimated to an even greater extent.

The scatter of cerebral oxygen uptake values in the group of young normal adults is of some theoretical interest. In the present series, the standard deviation of extrapolated bilateral $CMRO_2$ values was 0.2 cc per 100 g per minute or 7 per cent of the mean $CMRO_2$ of 3.0 cc per 100 g per minute. The same value was found in a previous study using the same bilateral technique (26). In order to compare this value to that of unilateral series the bilateral standard deviation must be corrected for the greater number of analyses involved, and a factor of $1/0.87 = 1.15$ may appropriately be used (26). The resulting corrected standard deviation of about 0.25 cc per 100 g per minute is significantly lower than that of about 0.5 cc per 100 g per minute reported in various unilateral series utilizing both the Kr^{85} method (9, 26), and the nitrous oxide method (18–20). On this basis, it may be concluded that the coefficient of variation of the random experimental error of determining cerebral blood flow and oxygen uptake in normal man is of the order of 7 per cent or less when the present bilateral method is employed. A maximum estimate of the random experimental error may also be obtained from the results of repeated bilateral studies. Combining the results of the three subjects in the present study with five cases reported previously (26), a coefficient of variation of 6 per cent is obtained.

The coefficients of variation mentioned above suggest a remarkable constancy of cerebral oxygen uptake; this parameter varies little when the same subject is studied repeatedly over a period of months. Moreover, in young normal adults no marked inter-individual differences seem to exist.

Before commenting on the results obtained in the two aged groups, it is worth noting that the side-to-side differences of cerebral blood flow and oxygen uptake (average 14 and 19 per cent, respectively) are in agreement with previous findings in bilateral studies (cf. References 1 and 26, and the discussion in 27).

Normal old age. The aged normal subjects had nearly the same average CBF as the young normal subjects, while the average bilateral $CMRO_2$ (infinity value) was 9 per cent lower than in young normal subjects. This decrease of $CMRO_2$ was statistically significant ($p < 0.05$). Other investigators, using unilateral techniques, have found a moderate (21, 22) to severe (3) reduction of $CMRO_2$ with age. However, the criteria of normality adopted in these studies are

not explicitly stated, and it is possible that the aged groups studied may have included some subjects whom we would have classified as deteriorated.

A study of $CMRO_2$ in young and aged normal subjects was recently completed in this laboratory by Sokoloff (28) and Dastur and associates (29). These investigators found no significant reduction of $CMRO_2$ in their aged group. However, their methods differed in that they used the unilateral N_2O technique without extrapolation, and their criteria of normality were more stringent than our own. (They would have rejected O_3 and O_5 solely on the basis of having been hospitalized.) For these reasons, this study cannot be directly compared with the present report.

Considering our own findings in the light of the evidence available in the literature, we would suggest that a small but definite reduction in $CMRO_2$ per 100 g of brain tissue occurs in normal persons of age 70 or more. A reduction in brain weight, not measurable with the present technique, would produce a still greater reduction in total cerebral oxygen uptake. We would further suggest that this reduction in $CMRO_2$ represents a physiological correlate of the well established decline in intellectual function with advanced age. Four out of five of the present group of aged normal subjects spontaneously reported recent changes in their intellectual function, primarily difficulties in remembering names and recent events. While such changes cannot be demonstrated with the tests employed, they are nevertheless quite real and are consistent with the interpretation of a mild degree of chronic brain syndrome or, perhaps, "incipient dementia."

The pathoanatomical basis of the metabolic and mental deterioration in advanced age is presumed to be a degenerative process which diffusely involves the cerebral cortex (cortical atrophy). This interpretation is consonant with the reduction in $CMRO_2$ (see the discussion in Reference 8). While these interpretations are by no means novel, what is new is the suggestion that $CMRO_2$ may represent an objective and quantitative measure of certain major cerebral changes in old age.

Organic dementia. The patients with organic dementia had bilateral cerebral oxygen uptake values which were markedly lower than those found in young normal subjects. The separation of the demented subjects from the aged normal control subjects was not as sharp; however, the latter may be considered to be cases of borderline chronic brain syndrome according to the above discussion. The various observations in the group of mentally deteriorated patients gave no indication of the etiology of the presumably degenerative cerebral affliction causing the fully developed chronic cerebral syndromes. As noted in the description of the patient material, no clinical clues existed either. There was, in particular, no reason to suspect cerebral vascular disease to be of general etiological importance. For a more extensive discussion of the etiological role of cerebral arteriosclerosis in organic dementia the reader is referred to a recent review (27). Thus it is possible that the group of senile demented patients were actually suffering from cerebral involutive processes of the same nature, but of more pronounced intensity, as those occurring in normal old age.

Disregarding etiological considerations, however, there seems to be no reason to emphasize the *age* of the mentally deteriorated subjects. The results obtained by Lassen, Munck and Tottey (8) in a group of middle-aged demented subjects of average age 55 years (range 42 to 64 years) are very similar to those obtained in the present group of senile patients of average age 74 years (range 67 to 84 years). Thus both studies found severe mental impairment associated with a 40 per cent reduction in cerebral oxygen uptake, whereas less pronounced degrees of dementia were associated with a 20 per cent reduction in cerebral oxygen uptake. It is likely that a reduction in brain weight occurred in many of the patients in both studies, reducing *total* cerebral oxygen uptake even more than is indicated by the inert gas studies.

The fairly close correlation between degree of dementia and reduction of the average rate of cerebral oxygen uptake of the whole brain suggests that intellectual processes involve the function of large parts of the hemispheres. When correlating the $CMRO_2$ to the raw scores of the psychometric tests, the unilateral values on the left side showed as high a correlation as the bilateral values, whereas no significant correlation was noted with the unilateral values of the right

side. This finding is presented more extensively elsewhere (30). It may signify some degree of gross localization of cognitive function, so that a left-sided cerebral lesion is of greater consequence for the development of dementia than is a comparable right-sided lesion.

SUMMARY

Cerebral oxygen uptake was determined in 11 young normal adults, 5 aged normal subjects, and 9 aged subjects suffering from organic dementia. The bilateral Kr^{85} modification of the inert gas method was employed. Mental function was assessed in the two aged groups by means of psychiatric interview and psychometric tests. The following results were obtained:

1. In the young normal adults the cerebral oxygen uptake was in average 3.5 cc O_2 per 100 g of brain per minute when the values were calculated corresponding to 10 minutes of inert gas inhalation. This result is in agreement with the findings of previous investigators. However, employing extrapolation to infinity, a mean value of 3.0 cc O_2 per 100 g per minute was found. Similar differences between 10 minute and extrapolated values were found in the two aged groups. The causes of these differences were discussed.

2. The aged normal subjects had an average age of 72 years and an average cerebral oxygen uptake which was 9 per cent below the level in young normal subjects ($p < 0.05$). This small reduction in cerebral oxygen uptake is considered to be a metabolic manifestation of an incipient degenerative cerebral disorder whose functional expression is the decline in mental function in normal subjects with advanced age.

3. The nine subjects with organic dementia (average age 74 years) had a cerebral oxygen uptake which was significantly reduced below the level in the young and aged control groups. The two subjects with the greatest degree of mental impairment also had the lowest values for cerebral oxygen uptake—values about 40 per cent below the level in young normal adults. The seven subjects with less severe mental defects had a more moderate reduction in cerebral oxygen uptake—in average, about 20 per cent below the level in young normal adults.

4. The reduction of cerebral oxygen uptake is considered to be a rough quantitative measure of cerebral degenerative lesion revealed functionally by the dementia.

ACKNOWLEDGMENT

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CORRECTION

Through an error in the editorial office, on page 2052 of the paper, "Chronic Progressive Myopathy with Myoglobinuria: Demonstration of a Glycogenolytic Defect in the Muscle," by Rudi Schmid and Robert Mahler (J. clin. Invest. 1959, **38**, 2044), PAS, periodic acid-Schiff reaction, was incorrectly defined as para-aminosalicylic acid.

PROTEIN-BINDING OF INORGANIC PHOSPHATE IN PLASMA OF NORMAL SUBJECTS AND PATIENTS WITH RENAL DISEASE*

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Despite a large amount of experimental data, it remains uncertain whether all of the plasma phosphate is filtrable through semipermeable membranes. Early micropuncture studies in Amphibia indicated that the concentration of inorganic phosphate in glomerular filtrate was the same as that in whole plasma (1, 2). If this is the case in mammals, approximately 17 per cent of the plasma phosphate may be nonfilterable. This follows from consideration of the normal plasma water content, 930 g per L, and the theoretical Donnan ratio for divalent anions, 0.90 to 0.91 (Reference 3, page 771). Since 80 per cent of the plasma phosphate consists of HPO_4^{2-} and 20 per cent $H_2PO_4^-$, the effective Donnan factor for plasma phosphate should be 0.91 to 0.92. The concentration of phosphate in ultrafiltrate would therefore be $1/0.93 \times 0.915$ or 1.175 times that in whole plasma.

Some of the pertinent reports are summarized in Table I. This list is not intended to be complete, and in particular omits the earliest observations, reported prior to 1926. In those instances in which the plasma or serum values were reported without correction for water content, a correction was applied as described below. The ratio of phosphate concentration in ultrafiltrate or dialysate to that in plasma or serum water for the entire group averages 0.94. The theoretical value, representing complete filtrability, is 1.09. Only a few isolated samples have been found to show ratios of 1.09 or higher.

The only two studies in which the pH and temperature were the same as that of blood *in vivo*, References 6 and 8, revealed mean ratios of 0.85 and 0.89, respectively. These studies both utilized measurements of saline solutions equilibrated with the circulating plasma.

It has also been reported that the filtrability of plasma phosphate may become markedly reduced when either plasma calcium or plasma phosphate is increased (5, 9, 11). These observations have supported the concept of a nondiffusible calcium phosphate complex. However, this substance is thought to be present only in abnormal plasma.

Toribara, Terepka and Dewey (13) have shown that the filtrability of plasma calcium is influenced by the pH and temperature at which ultrafiltration is performed. Phosphate analyses of ultrafiltrates prepared at body temperature and normal plasma pH have not been reported. The reported data (Table I) are for the most part based upon small series and exhibit considerable scatter. The filtrability of phosphate in uremic plasma has apparently not been studied. The occurrence of a "colloidal" calcium phosphate complex might be anticipated under these conditions, since hyperphosphatemia is often pronounced.

For these reasons, ultrafiltration was carried out at 37° C in an atmosphere of 5 per cent CO_2 and 95 per cent O_2, using plasma samples from normal subjects and from patients with azotemia. The results indicate that a significant fraction of the plasma inorganic phosphate is apparently bound to proteins. This binding is not dependent upon plasma calcium or magnesium. In azotemic patients without phosphate retention, protein-binding of phosphate is the same as in normal subjects. In the presence of phosphate retention, no evidence was found for a decrease in the filtrability of phosphate. On the contrary, the percentage of phosphate bound to protein did not differ significantly from normal.

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TABLE I

Ratios of the concentration of phosphate in ultrafiltrate or dialysate to that in plasma or serum water in reported studies on normal humans and dogs

Authors	No. and species	Method	Range	Mean
Pincus *et al.* (4) 1926	6 Dogs	Ultrafiltration	0.90–1.10	1.01
Grollman (5) 1927	7 Dogs	Ultrafiltration	1.00–1.00	1.00
Greene and Power (6) 1931	14 Dogs	Dialysis *in vivo*	0.54–1.11	0.85
Benjamin and Hess (7) 1933	21 Humans	Ultrafiltration	Not given	0.97
	73 Dogs	Ultrafiltration	Not given	0.94
	25 Rats	Ultrafiltration	Not given	0.93
Cantarow and Haury (8) 1939	6 Dogs	Vividiffusion	0.85–0.95	0.89
Harrison and Harrison (9) 1941	6 Dogs	Ultrafiltration	0.87–0.93	0.91
Smith *et al.* (10) 1943	13 Humans	Ultrafiltration	0.97–1.08	0.96
Hopkins *et al.* (11) 1952	6 Humans	Ultrafiltration	0.90–1.09	0.99
Foulks (12) 1955	5 Dogs	Ultrafiltration	0.79–0.88	0.85

METHODS

Venous blood was obtained in oiled syringes wet with heparin solution, and separated without exposure to air. Ultrafiltration was carried out by the method of Toribara and associates (13). Plasma pH, measured before and after ultrafiltration, did not change more than 0.05 unit. Phosphate was determined on trichloroacetic acid filtrates of plasma and on ultrafiltrates by the method of Fiske and Subbarow (14). The recovery of phosphate added to plasma or ultrafiltrate averaged 100 per cent. Protein determinations were performed by the biuret method (15). Plasma water content, in grams per liter, was approximated as 990 minus 8 [Prot], where [Prot] represents the protein concentration in grams per cent (3).

In order to determine whether equilibrium across the cellophane membrane persisted during ultrafiltration, several types of experiments were performed. *1)* Phosphate concentrations were compared in the fluid formed during the first half hour of ultrafiltration, representing approximately 15 per cent of the volume of plasma used, and during the ensuing three hours, at the end of which the volume of plasma remaining was about one-half of the original volume. In four normal samples, the ratios of phosphate concentrations in the first portion to the last portion were 0.99, 0.97, 0.98, and 0.97. *2)* Ultrafiltrates of normal plasma were subjected to ultrafiltration again, one-half of the total volume passing through the casing. The concentration of phosphate in the newly formed ultrafiltrate was, on the average, 3 per cent less than that in the original ultrafiltrate. *3)* Ultrafiltrates were prepared from a solution of sodium phosphate, 0.061 M, at pH 7.4, and also from this solution diluted 20-fold with isotonic saline. Again, centrifugation was continued until one-half of the total volume used had passed out of the sac. The phosphate concentration in these ultrafiltrates was, on the average, 2 per cent lower than in the original solutions. *4)* Normal plasma samples were dialyzed against their own ultrafiltrates. This was done by filling a sac prepared from Visking casing with plasma obtained anaerobically, plus a small amount of mercury. This sac was inserted into a tube containing an equal volume of ultrafiltrate, plus a small amount of mercury. The tube was closed with a stopper, into which a needle was inserted in order to remove entrapped air. The needle was removed, and the tube was then rocked mechanically in a constant temperature bath at 37° C for six hours. The concentration of phosphate in ultrafiltrates of normal plasma was unchanged by this process.

All of the above results lend support to the conclusion that the method of ultrafiltration yields a valid sample of fluid in equilibrium with whole plasma. Deviations from equilibrium are so small that they lie within the experimental error.

RESULTS

The results of 24 determinations performed in 15 normal subjects and in 4 normal dogs are shown in Table II.

The ratio of phosphate concentration in ultrafiltrate to that in plasma water in the human sub-

jects averaged 0.825. As indicated above, if all of the phosphate were ultrafilterable, the ratio should be 1:0.915 or approximately 1.09. The filtrable inorganic phosphate of plasma therefore comprises 0.825: 1.09 or 76 per cent of the total.

The assumption that ultrafiltrate phosphate equals plasma phosphate entails only a small error, because it ignores corrections for Donnan factor and water content. In this series of subjects, the ratio of ultrafiltrate phosphate to plasma phosphate (uncorrected for water content) averages 0.89.

In Table III are shown the results of observations in a series of patients with renal disease. Although all of these subjects had signs of renal damage, in many the degree of azotemia was slight. Consequently phosphate retention was present in only 21 of the 32 patients. The highest plasma phosphate observed was 5.32 mmoles per L or 16.5 mg per 100 ml.

In the patients without phosphate retention, the ratio of ultrafiltrate phosphate to plasma water phosphate averaged 0.866, a value which does not differ significantly from the normal mean of 0.825. In the patients with phosphate retention, this ratio averaged 0.889, which also is not significantly greater than the normal mean. Thus the absolute quantity of phosphate bound is increased approximately in proportion to the rise in plasma phosphate. For example, in the five plasma samples containing 3 to 4 mmoles per L of phosphate, the average filtrate phosphate is 3.14 mmoles per L. Applying a Donnan factor of 0.915, the free phosphate in plasma water becomes 2.87 mmoles per L. Total phosphate in plasma water averages 3.32 mmoles per L. Therefore bound phosphate is 3.32 to 2.87 or 0.45 mmoles per L, or 14 per cent of the total. In the normal subjects similar calculation reveals that an average of 0.31 mmoles per L, or 25 per cent, of the total phosphate is bound. The differ-

TABLE II

*Protein-binding of inorganic phosphate (normal subjects)**

	Subject	$[Protein]_P$	$[Pi]_P$	$[Pi]_{PW}$	$[Pi]_{UF}$	Ratio $[Pi]_{UF}/[Pi]_{PW}$
		gm %	*mmoles/L*	*mmoles/L*	*mmoles/L*	
Normal dogs	A	6.4	1.89	2.02	1.61	0.80
	B	6.6	1.18	1.26	1.15	0.91
	C	6.2	1.26	1.34	1.29	0.96
	D	6.7	1.57	1.68	1.24	0.74
					Mean	0.85
Normal men	MW	6.5	0.74	0.79	0.69	0.87
	MW	7.2	1.15	1.23	0.96	0.78
	MW	7.2	1.13	1.21	0.96	0.78
	MW	6.6	1.22	1.30	0.98	0.75
	IW	7.2	0.79	0.85	0.74	0.75
	IW	6.9	0.88	0.94	0.84	0.89
	GM	7.6	1.43	1.54	1.38	0.90
	DS	7.7	1.04	1.12	1.00	0.89
	LL	6.5	1.30	1.39	1.15	0.83
	JB	6.3	1.52	1.62	1.44	0.89
	KG	6.5	1.07	1.14	1.09	0.96
Normal women	GK	7.6	0.96	1.03	0.80	0.78
	GK	6.6	0.99	1.06	0.86	0.81
	JS	8.0	1.38	1.49	1.04	0.70
	JS	6.3	1.28	1.36	1.22	0.90
	MF	7.1	1.14	1.22	1.04	0.85
	BC	7.2	1.26	1.35	1.03	0.76
	MS	8.9	1.21	1.33	0.97	0.73
	RC	6.5	1.45	1.55	1.27	0.82
	MM	6.6	0.88	0.94	0.76	0.81
	SB	7.3	1.16	1.24	1.07	0.86
					Mean	0.825 ± 0.066(SD)

* $[Pi]_P$ = inorganic phosphate concentration in plasma; $[Pi]_{PW}$ = inorganic phosphate concentration in plasma water; $[Pi]_{UF}$ = inorganic phosphate concentration in plasma ultrafiltrate.

TABLE III

*Protein-binding of inorganic phosphate in patients with azotemia**

	Subject	[Protein]$_P$	[Pi]$_P$	[Pi]$_{PW}$	[Pi]$_{UF}$	Ratio [Pi]$_{UF}$/[Pi]$_{PW}$
		gm %	*mmoles/L*	*mmoles/L*	*mmoles/L*	
Patients without phosphate retention	Jon	6.6	1.07	1.14	0.99	0.87
	Gre	6.5	1.12	1.20	1.03	0.86
	Si	7.3	1.27	1.36	1.19	0.88
	Bar	7.4	1.32	1.42	1.27	0.89
	Sim	7.0	1.33	1.42	1.30	0.92
	Ste	7.3	1.43	1.53	1.32	0.86
	Wil	6.2	1.41	1.50	1.26	0.84
	Dil	6.6	1.46	1.56	1.28	0.82
	Kin	7.8	1.45	1.56	1.41	0.90
	Hew	8.1	1.49	1.61	1.26	0.78
	Spe	6.6	1.54	1.64	1.50	0.91
					Mean	0.866 ± 0.041(SD)
Patients with phosphate retention	New	5.5	1.61	1.70	1.47	0.86
	Sta	5.9	1.67	1.77	1.60	0.90
	Wil	7.0	1.71	1.85	1.57	0.85
	Edw	6.0	1.75	1.86	1.71	0.92
	Mor	5.7	1.71	1.81	1.52	0.84
	Lin	6.3	1.82	1.94	1.74	0.90
	Jac	5.7	1.81	1.91	1.78	0.93
	Sta	6.8	1.80	1.92	1.64	0.86
	Bro	6.6	1.80	1.92	1.68	0.88
	Har	6.2	1.97	2.10	1.80	0.86
	Fis	5.7	2.05	2.17	1.92	0.88
	Ree	4.3	2.28	2.38	2.07	0.87
	Dav	6.6	2.43	2.59	2.30	0.89
	Goo	5.7	2.79	2.95	2.57	0.87
	Wav	6.9	3.02	3.23	3.05	0.94
	Edw	5.7	3.14	3.32	3.05	0.92
	Jac	5.8	3.18	3.38	3.03	0.90
	She	6.2	3.48	3.70	3.36	0.91
	Dis	5.5	3.44	3.64	3.08	0.84
	Seg	6.4	5.07	5.39	5.23	0.97
	Hie	7.4	5.32	5.72	5.14	0.90
					Mean	0.889 ± 0.034(SD)

* Symbols as in Table II.

ence in protein concentration between the two groups was minor.[1]

In order to determine whether some form of interaction with calcium is involved in the apparent protein-binding of phosphate, the trisodium salt of ethylenediaminetetraacetic acid (EDTA) was added to plasma before ultrafiltration. Five ml of plasma was mixed with 0.2 ml of EDTA solution, (adjusted to pH 7.4) and ultrafiltered. This amount of EDTA is sufficient to complex the entire amount of calcium and magnesium present. The concentration of phosphate in this ultrafiltrate was compared to that in ultrafiltrate from the same plasma diluted with saline instead of EDTA solution. No difference was noted in three experiments. These results indicate that the protein-binding of phosphate in normal plasma is not dependent upon the presence of calcium or magnesium.

DISCUSSION

The results of this study indicate that a significant portion of the inorganic phosphate of plasma is not filtrable through cellophane. In normal subjects, this moiety amounts to about one-fourth of the total plasma inorganic phosphate; in patients with renal disease and phosphate retention, it is a somewhat smaller fraction. The probable explanation of these findings is protein-binding. It is also conceivable that phosphate might exist in plasma in a nondiffusible form without being

[1] It is possible that hyperlipemia, with a consequent decrease in plasma water content, might account for part of the apparent increase in this ratio in renal failure. However, none of the subjects had the nephrotic syndrome or frankly lipemic plasma.

bound to protein, calcium or magnesium. However, there is no evidence for such a compound at present. Govaerts (16) reported that a portion of the plasma phosphate does not exchange readily with administered radiophosphate. As Handler and Cohn (17) have shown, the specific activity of urinary phosphate is equal to that of plasma phosphate during a constant infusion of radiophosphate. These authors suggested that urinary delay time, or exchange with a pool of inorganic phosphate in the tubular epithelium, may account for the findings of Govaerts. It is also possible, however, that the exchange of administered radiophosphate with protein-bound phosphate may be significantly slower than with the filtrable portion of plasma phosphate.

We have not investigated the filtrability of phosphate in the presence of hypercalcemia. Others (5, 11) have reported that a nonfiltrable calcium phosphate complex is formed under these conditions. In normal plasma, the data presented here indicate that the protein-binding of phosphate is not dependent upon the presence of calcium or magnesium.

The present results probably do not alter the conclusions of the numerous studies pertaining to renal tubular reabsorption of phosphate in man and dogs. In most of these studies, it has been assumed that phosphate concentration in the glomerular filtrate is equal to phosphate concentration in plasma water. As indicated above, this assumption entails an error of 15 to 20 per cent in normal subjects, and less in patients with renal disease. The possibility also remains that the filtrability of phosphate at the glomerulus may differ from that indicated by cellophane membranes.

The theoretical Donnan ratio, $1/r^2$, as calculated by Van Slyke, Wu and McLean (3), is approximately 1.10 at normal pH and protein concentration. This is the factor which has been employed herein, in order to estimate the free phosphate ion concentration in whole plasma. As Manery (18) has pointed out, the observed Donnan ratios for various ions in plasma are all less that this theoretical value. The deviation is very small in the case of sodium and potassium but greater in the case of chloride, sulfate (19), ferrocyanide (20) and phosphate. Purified proteins bind virtually all anions which have been studied (21, 22). It seems reasonable to infer, therefore, that free phosphate ions in plasma are distributed across semipermeable membranes in accordance with the theoretical factor, and that the remainder of plasma phosphate is bound to protein.

SUMMARY

Ultrafiltrates were prepared at 37° C in an atmosphere of 5 per cent CO_2 from plasma samples obtained from 4 dogs, 15 normal men and women, and from 32 patients with varying degrees of azotemia. In the normal subjects, an average of one-fourth of the inorganic phosphate of plasma was found to be protein-bound. This binding was not dependent upon the presence of calcium or magnesium.

In patients with azotemia, with or without phosphate retention, the fraction of plasma inorganic phosphate bound to protein was not significantly different.

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THE PHYSIOLOGICAL SIGNIFICANCE OF THE SECRETION OF ENDOGENOUS INSULIN INTO THE PORTAL CIRCULATION. III. EVIDENCE FOR A DIRECT IMMEDIATE EFFECT OF INSULIN ON THE BALANCE OF GLUCOSE ACROSS THE LIVER* †

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There is at present general agreement, based upon abundant *in vitro* and *in vivo* experimental evidence, that insulin exerts an immediate and profound effect on glucose utilization by muscle and adipose tissue (1–5). On the other hand, a direct hepatic action of insulin is disclaimed by most investigators since innumerable studies both *in vitro* and *in vivo* have failed to demonstrate clearly a consistent and reproducible effect of insulin upon hepatic glucose metabolism (5–12).

Recent observations from this laboratory suggested that the site (13) and the rate (14) of insulin administration are important factors in eliciting a hepatic effect. However, in these as in all previous *in vivo* studies (11, 15–19), the conclusions regarding the hepatic action of insulin were inferential, since the net balance of glucose across the liver was not ascertained by direct measurement.

Any experiments designed to elucidate the effect of insulin on hepatic carbohydrate metabolism should fulfill the following prerequisites:

1. Hepatic, in contrast to splanchnic, glucose metabolism should be measured. In view of the marked sensitivity of the extrahepatic splanchnic tissues to insulin (1–5, 11, 12), separation of the effects of insulin on these tissues from its effects on the liver is mandatory. This, in turn, requires the measurement not only of arterial, portal venous and hepatic venous glucose concentration but also measurement of the precise contributions of portal venous and hepatic arterial inflow to total hepatic blood flow. This is not possible in the intact animal because of the wide and capricious variation in the contribution of portal venous inflow to total hepatic blood flow, which may change from as little as 10 per cent to as much as 90 per cent of total hepatic blood flow (20).

2. Insulin should be administered in a manner which minimizes the counter-regulatory response to hypoglycemia. Profound hypoglycemia with its attendant release of epinephrine (21, 22), adrenal cortical hormones (23, 24), and glucagon (25, 26), may obscure a hepatic effect of insulin by the marked increase in hepatic glucose release which characterizes the action of these counter-regulatory hormones (27–30).

The present experiments were designed to meet these prerequisites in the following manner. First, dogs with complete end-to-side portacaval shunts were studied. This operation completely separates the liver from the remainder of the splanchnic tissues and thereby permits the measurement of hepatic rather than splanchnic glucose metabolism. Second, measures were taken to minimize or prevent the counter-regulatory mechanisms to hypoglycemia. In one group of experiments, insulin was administered by slow infusion which produced a very gradual modest decline in arterial glucose concentration. In another group, the hypoglycemic stimulus was further reduced in magnitude and duration by administering glucose after insulin infusion had been started. Finally hypoglycemia was prevented by using diabetic dogs with fasting hyperglycemia.

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METHODS AND PROCEDURE

Complete end-to-side portacaval anastomoses were performed in one stage on adult mongrel dogs under general ether anesthesia. A minimal period of two weeks after portacaval surgery was allowed to pass prior to the performance of the experimental studies. By this time, the dogs had completely recovered from the operative procedure. Food was removed from the cages 15 hours before the dogs were anesthetized with Nembutal (25 mg per kg intravenously), which has been shown to alter neither hepatic blood flow nor hepatic oxygen utilization (31, 32). Hepatic venous blood samples were collected through a cardiac catheter inserted into an external jugular vein and guided deep into a hepatic vein under fluoroscopic control. Position of the hepatic venous catheter was checked frequently during each experiment. Arterial samples were obtained through an indwelling Cournand needle placed in a femoral artery.

Hepatic blood flow (EHBF) was estimated at 10 minute intervals by the clearance and extraction method of Bradley, Ingelfinger, Bradley and Curry (33), using I^{131}-labeled rose bengal as the extractable material. The validity of the clearance and extraction method of estimating hepatic blood flow in the presence of a portacaval shunt has been established by Bradley and co-workers (34). I^{131}-rose bengal has been shown to be a satisfactory substance for the measurement of hepatic blood flow (35). A solution of isotonic saline containing 20 μc of I^{131}-labeled rose bengal per 100 ml was administered intravenously at a constant rate of approximately 1 ml per minute by means of a Bowman pump. After one hour to allow for equilibration, 3 ml samples of blood were drawn simultaneously from the femoral artery and hepatic vein at 10 minute intervals. Each sample was hemolyzed with powdered saponin and the radioactivity contained in duplicate 1 ml aliquots of the hemolyzed blood was measured in a deep-well scintillation counter. The coefficient of variation of the mean of pair determinations of I^{131}-rose bengal from a group average is 0.52 per cent (36). Hepatic extraction of I^{131}-rose bengal always exceeded the minimum criteria of Bradley and associates (34), most values being in the range of 15 to 30 per cent.

Hepatic blood flow was calculated from extrapolated data midway between two successive determinations of arterial and hepatic venous blood radioactivity by means of the formula:

$$\text{EHBF} = \frac{\text{I} \pm \Delta\text{A}_\text{C} \times \text{BV}}{\text{A}_\text{C} - \text{HV}_\text{C}},$$

where EHBF = estimated hepatic blood flow in milliliters per minute; I = infusion rate of I^{131}-labeled rose bengal in counts per minute per minute; ΔA_C = change in counts per milliliter of arterial blood per minute; BV = total blood volume in milliliters, estimated as 9 per cent of body weight; A_C = counts per minute per milliliter of arterial blood; and HV_C = counts per minute per milliliter of hepatic vein blood.

Midway between the sampling times for radioactivity, 4 ml of blood was drawn simultaneously, with adequate precaution to prevent catheter dead space error, and at a constant rate over a 100 second interval from the femoral artery and hepatic vein for determination of glucose. To minimize glycolysis the blood was placed immediately into iced tubes containing oxalate and sodium fluoride and protein-free filtrates were prepared within 10 minutes of collection. Blood glucose was determined in triplicate on each sample by the Somogyi copper iodometric method on 5 ml of blood filtrate prepared immediately from 3 ml blood samples, thereby providing a valid measurement of blood glucose within 1 mg per 100 ml (37, 38). Hepatic glucose output (HGO) in milligrams per minute at each 10 minute interval was calculated as the product of the estimated hepatic blood flow (EHBF) and the hepatic venous-femoral arterial glucose concentration difference (HV-A).

One group of experiments was designed to contrast the effects of the slow intravenous infusion and rapid intravenous injection of insulin on hepatic glucose metabolism. After three or four control determinations of hepatic glucose output, glucagon-free insulin [1] was administered into a hind leg vein either slowly by constant infusion at a rate of 0.025 to 0.08 unit per minute in eight studies,[2] or rapidly in amounts of 5 to 9 units over a period of 15 seconds in six studies. Hepatic glucose output was then measured at 10 minute intervals over the ensuing 60 to 90 minutes. In some studies, the constant infusion of insulin was stopped, and additional determinations were obtained during the "recovery" period.

Ten additional studies were performed under circumstances calculated either to prevent or to reduce the magnitude of the hypoglycemic stimulus. In six experiments, insulin was administered at a rate of 0.026 to 0.065 unit per minute by constant infusion in the usual manner. Thirty to 40 minutes after the start of the insulin infusion, glucose was added to the infusion and delivered at a rate of 25 to 200 mg per minute. Four other studies were performed in diabetic dogs with portacaval shunts. Diabetes was produced by the combination of subtotal pancreatectomy and alloxanization at least three weeks before each experiment. In these studies arterial glucose concentration fell from hyperglycemic levels during insulin infusion but never reached hypoglycemic levels.

RESULTS

1. Effect on hepatic glucose output

A. Slow infusion of insulin. Insulin administered by slow intravenous infusion resulted, in each of the eight dogs, in an immediate and sig-

[1] We are indebted to Dr. W. R. Kirtley of the Eli Lilly Company for the generous supply of glucagon-free insulin.

[2] See Tables I, III, IV and V for the precise dose of insulin in units per kilogram per hour.

TABLE I

Effect of the slow infusion of glucagon-free insulin upon hepatic glucose output

Dog		Control values*					Time during and after insulin infusion								
		−30	−20	−10	0	Mean control	10	20	30	40	50	60	70	80	90
No. 114	EHBF		218	200	174	197	188	201	168	193	173	178		212	184
19.6 kg	HV		110.2	109.6	115.5	111.7	108.2	100.9	84.0	80.9	82.3	75.7		66.3	62.3
	A		86.6	85.7	88.5	86.9	87.0	85.7	73.9	70.5	64.4	59.9		47.5	34.0
	HV-A		23.6	23.9	27.0	24.8	21.2	15.2	10.1	10.4	17.9	15.8		18.8	28.3
	HGO		51.4	47.8	47.0	48.7	39.9	30.6	17.0	20.1	31.0	28.1		39.9	52.1
	Insulin						← 0.05 *units/min* → (0.15 *units/kg/hr*)				←0.075 *units/min*→ (0.23 *units/kg/hr*)		None →		
No. 1028	EHBF	330		267	306	301	367	363	360	373	419	310			
18.2 kg	HV	72.2		96.4	86.9	85.1	80.6	75.4	70.2	67.6	62.5	61.8			
	A	57.0		67.5	67.3	63.9	64.6	63.0	61.3	59.3	55.1	52.0			
	HV-A	15.2		28.9	19.6	21.2	16.0	12.4	8.9	8.3	7.4	9.8			
	HGO	50.3		77.2	60.0	62.5	58.7	45.0	32.0	31.0	31.3	30.4			
	Insulin						← 0.05 *units/min* → (0.16 *units/kg/hr*)				←0.08 *units/min*→ (0.26 *units/kg/hr*)				
No. 103	EHBF	198	216	185	175	194	175	174	210	192	205	209	219	275	
14.5 kg	HV	80.6	87.7	84.4	84.7	84.4	78.3	69.6	66.3	59.1	52.8	50.1	41.8	38.0	
	A	69.3	73.7	73.4	68.2	71.2	66.0	63.8	60.0	53.1	47.3	42.4	33.3	28.9	
	HV-A	11.3	14.0	11.0	16.5	13.2	12.3	5.8	6.3	6.0	5.5	7.7	8.5	9.1	
	HGO	22.4	30.2	20.4	28.9	25.5	21.5	10.1	13.2	11.5	11.3	16.1	18.6	25.0	
	Insulin						← 0.04 *units/min* → (0.16 *units/kg/hr*)				←0.064 *units/min*→ (0.26 *units/kg/hr*)		None →		
No. 924	EHBF		272	296	288	285	315	295	296	304	303	300			
14.5 kg	HV		92.7	88.8	87.5	89.7	89.1	86.9	78.7	81.1	73.2	66.6			
	A		80.6	78.1	79.2	79.3	81.4	80.3	75.1	75.1	69.0	61.9			
	HV-A		12.1	10.7	8.3	10.4	7.7	6.6	3.6	6.0	4.2	4.7			
	HGO		32.9	31.7	23.9	29.5	24.3	19.5	10.7	18.2	12.7	14.1			
	Insulin						←0.036 *units/min*→ (0.15 *units/kg/hr*)			←0.058 *units/min*→ (0.24 *units/kg/hr*)					

* Abbreviations are as follows: EHBF, estimated hepatic blood flow in milliliters per minute; HV, hepatic venous glucose concentration in milligrams per 100 ml; A, arterial glucose concentration in milligrams per 100 ml; HV-A, hepatic venous minus arterial glucose concentration in milligrams per 100 ml; HGO, hepatic glucose output in milligrams per minute.

TABLE I—*Continued*

Dog		Control values*				Mean control	Time during and after insulin infusion								
		−30	−20	−10	0		10	20	30	40	50	60	70	80	90
No. 916	EHBF			244	311	278	340	353	331	335	345	310	418		
15.9 kg	HV			98.6	108.6	103.6	104.4	100.2	96.3	90.7	83.7	75.9	66.1		
	A			80.6	87.0	83.8	88.5	91.3	87.6	82.9	74.2	64.7	56.2		
	HV-A			18.0	21.6	19.8	15.9	8.9	8.7	7.8	9.5	11.2	9.9		
	HGO			43.9	67.2	55.6	54.1	31.4	28.8	26.1	37.5	34.7	41.0		
	Insulin						←0.027 *units/min*→ (0.10 *units/kg/hr*)			←0.047 *units/min*→ (0.18 *units/kg/hr*)					
No. 930	EHBF		226	215	231	224	199	238	236	221	248	296	313		
15.0 kg	HV		98.4	117.7	116.2	110.7	103.7	97.5	93.2	93.5	87.6	80.6	76.5		
	A		85.3	87.2	100.2	90.9	93.2	93.1	90.8	85.8	80.6	75.1	70.4		
	HV-A		13.1	30.5	16.0	19.8	10.5	4.4	2.4	7.7	7.0	5.5	6.1		
	HGO		29.6	65.5	37.0	44.1	20.5	10.5	5.7	17.0	17.4	16.3	19.1		
	Insulin						←0.038 *units/min*→ (0.15 *units/kg/hr*)			←0.06 *units/min*→ (0.24 *units/kg/hr*)					
No. 923	EHBF		263	271	278	271	289	304	328	341	340	231		233	200
15.2 kg	HV		109.2	112.5	106.5	109.4	106.9	90.7	79.9	74.5	73.8	74.7		84.1	93.3
	A		79.2	87.9	84.9	84.4	84.8	79.9	75.1	72.7	67.1	66.1		60.4	61.9
	HV-A		30.0	24.6	21.6	25.4	22.1	10.8	4.8	1.8	6.7	8.6		23.7	31.4
	HGO		78.9	66.7	59.4	68.3	63.8	32.8	15.7	6.1	22.8	19.8		55.3	62.8
	Insulin						←0.025 *units/min*→ (0.10 *units/kg/hr*)						None→		
No. 919	EHBF		206	174	172	184	174	175	209	217	222	204	257	250	303
15.0 kg	HV		92.3	94.0	90.4	92.2	84.5	81.9	77.5	69.8	64.7	55.3	51.2	44.9	42.7
	A		77.8	78.6	77.8	78.0	75.5	74.3	71.5	63.8	58.6	49.6	43.8	37.5	35.1
	HV-A		14.5	15.4	12.6	14.2	9.0	7.6	6.0	6.0	6.1	5.7	7.4	7.4	7.6
	HGO		29.9	26.8	21.7	26.1	15.6	13.3	12.5	13.0	13.5	11.6	19.0	18.5	23.0
	Insulin						←0.04 *units/min*→ (0.16 *units/kg/hr*)						←0.064 *units/min*→ (0.25 *units/kg/hr*)		
Mean	EHBF		234	232	242	241	256	263	267	272	282	255			
16.0 kg	HV		98.4	100.3	99.5	98.4	94.5	87.9	80.8	77.1	72.5	67.6			
	A		80.5	80.0	81.6	79.8	80.1	78.9	74.4	70.4	64.5	59.0			
	HV-A		17.9	20.3	17.9	18.6	14.4	9.0	6.4	6.7	8.0	8.6			
	HGO		42.2	47.5	43.1	45.0	37.3	24.2	17.0	17.9	22.2	21.4			
	Insulin						←0.038 *units/min*→ (0.14 *units/kg/hr*)			←0.056 *units/min*→ (0.20 *units/kg/hr*)					

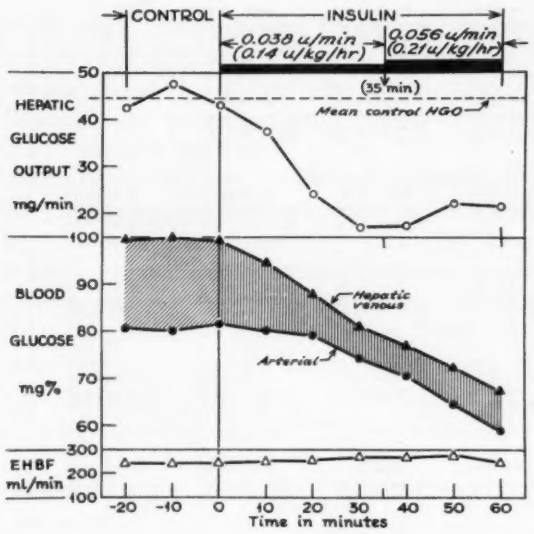

FIG. 1. MEAN CHANGES IN HEPATIC VENOUS AND ARTERIAL GLUCOSE CONCENTRATIONS, HEPATIC BLOOD FLOW, AND THE HEPATIC GLUCOSE OUTPUT DURING THE SLOW INFUSION OF GLUCAGON-FREE INSULIN.

nificant (36) decline in hepatic glucose output (Tables I and II). The 44 per cent fall in mean hepatic glucose output, from the control value of 45 mg per minute to the mean value of 25.3 mg per minute during the 60 minute period of insulin infusion, was attributed to a 52 per cent decrease in mean HV-A glucose difference which fell from 18.6 mg per 100 ml to 8.8 mg per 100 ml (Figure 1). During the last 30 minutes of insulin infusion, hepatic glucose output and HV-A glucose difference averaged 19.8 mg per minute and 7.4 mg per 100 ml, a fall of 56 and 60 per cent, respectively, from control values (Figures 1 and 2).

TABLE II

Changes in hepatic glucose output from control during the slow infusion of insulin

Dog no.	Mean control	Hepatic glucose output, mg/min: Decrease from control, Minutes after start of insulin infusion 10	20	30	40	50	60
114	47.8	7.9	10.1	30.8	27.7	16.8	19.7
1028	62.5	3.8	17.5	30.5	31.5	31.2	32.1
103	25.5	4.0	15.4	12.3	14.0	14.2	9.4
924	29.5	5.2	10.0	18.8	11.3	16.8	15.4
916	55.6	1.5	24.2	26.8	29.5	18.1	20.9
930	44.1	23.6	33.6	38.4	27.1	26.7	27.8
923	68.3	4.5	35.5	48.1	62.2	45.5	48.5
919	26.1	10.5	12.8	13.6	13.1	12.6	14.5
Mean		7.6	19.9	27.4	27.0	22.7	23.5
p		<0.02	<0.01	<0.01	<0.01	<0.01	<0.01

B. Rapid intravenous injection of insulin. In contrast to the immediate and striking decrease in hepatic glucose output associated with the slow infusion of insulin, the rapid intravenous injection of insulin did not decrease hepatic glucose output (Table III). In no instance did hepatic glucose output fall significantly during the first 40 minutes after rapid insulin injection when mean arterial blood glucose concentration was continuously falling. During this time hepatic glucose output averaged 51.0 mg per minute, a 21 per cent increase over the control value. From

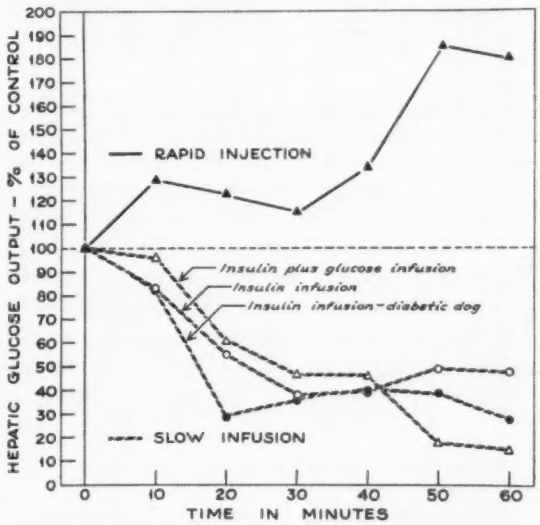

FIG. 2. COMPARISON OF THE MEAN CHANGES IN HEPATIC GLUCOSE OUTPUT FOLLOWING RAPID INTRAVENOUS INJECTION AND DURING THE SLOW INTRAVENOUS INFUSION OF GLUCAGON-FREE INSULIN.

40 to 70 minutes, when arterial blood glucose stopped falling and rose slightly, mean hepatic glucose output was 72.3 mg per minute, a value 74 per cent above control (Figures 2 and 3).

C. Studies in which hypoglycemia was minimized or prevented. In the diabetic dogs with portacaval shunts, mean hepatic glucose output decreased 51 per cent from the control value of 76.8 mg per minute to a mean value of 37.9 mg per minute during the 60 minute period of insulin infusion (Table IV). During this time, HV-A glucose difference fell 65 per cent from 24.8 to 8.7 mg per 100 ml. From 30 to 60 minutes after

TABLE III

Effect of the rapid intravenous injection of glucagon-free insulin in hepatic glucose output

Dog		Control values*					Time after rapid intravenous injection of glucagon-free insulin								
		−30	−20	−10	0	Mean control	10	20	30	40	50	60	70	80	90
No. 316	EHBF		218	221	215	218	223	229	261	223	260	208	282	240	
♀	HV		95.8	93.0	85.7	91.5	86.9	74.9	62.9	54.8	60.4	62.4	49.8	54.8	
17.0 kg	A		82.7	81.3	78.5	80.8	72.7	61.5	50.7	42.0	41.8	43.2	37.0	37.0	
	HV-A		13.1	11.7	7.2	10.7	14.2	13.4	12.2	12.8	18.6	19.2	12.8	14.8	
	HGO		28.6	25.9	15.5	23.3	31.7	30.7	31.8	28.5	48.4	39.9	36.1	35.5	
	Insulin						←6 units								
No. 217	EHBF		165	165	168	166	151	142	134	126	96	99	137		
♂	HV		55.3	54.5	51.6	53.8	49.3	47.0	45.0	42.7	42.4	41.8	39.0		
10.1 kg	A		48.1	46.4	42.4	45.6	39.0	37.8	35.8	30.0	30.4	26.9	28.9		
	HV-A		7.2	8.1	9.2	8.2	10.3	9.2	9.2	12.7	12.0	14.9	10.1		
	HGO		11.9	13.4	15.5	13.6	15.6	13.1	12.3	16.0	11.5	14.8	13.8		
	Insulin						←5 units								
No. 210	EHBF		539	560	550	550	534	514	538	552	482	490	586	518	
♂	HV		77.3	77.9	74.1	76.4	62.1	52.4	39.5	33.7	48.3	48.0	50.1	48.0	
22.3 kg	A		71.5	72.1	69.4	71.0	57.1	47.2	34.0	29.3	36.3	37.0	38.7	40.1	
	HV-A		5.8	5.8	4.7	5.4	5.0	5.2	5.5	4.4	12.0	11.0	11.4	7.9	
	HGO		31.3	32.5	25.8	29.9	26.7	26.7	29.6	24.3	57.8	53.9	66.8	40.9	
	Insulin						←7 units								
No. 424	EHBF	408	433	459	416	429	612	609	606	654	632	523	364	446	370
♂	HV	98.7	90.5	84.8	84.0	89.5	77.2	61.1	53.2	50.7	67.4	69.0	91.1	90.3	96.0
34.0 kg	A	87.3	82.9	77.7	73.6	80.4	62.4	47.4	38.2	35.5	43.4	46.1	54.8	62.7	62.7
	HV-A	11.4	7.6	7.1	10.4	9.1	14.8	13.7	15.0	15.2	24.0	22.9	36.3	27.6	33.3
	HGO	46.5	32.9	32.6	43.3	38.8	90.6	83.4	90.9	99.4	151.7	119.8	132.1	123.1	123.2
	Insulin						←9 units								
No. 429	EHBF	275	272	343	377	317	342	356	374	367	339	311	383	339	278
♂	HV	109.3	113.9	113.1	117.4	113.4	110.9	95.7	69.1	71.6	79.7	91.6	95.2	113.1	87.3
25.5 kg	A	82.7	82.3	84.0	89.2	84.7	80.0	64.8	48.5	42.0	41.2	43.1	43.3	47.4	46.4
	HV-A	26.6	30.7	29.1	28.2	28.7	30.9	30.9	20.6	29.6	38.5	48.5	52.9	65.7	40.9
	HGO	73.2	83.5	99.8	106.7	90.6	105.7	110.0	77.0	108.6	130.5	150.8	202.7	222.7	113.7
	Insulin						←7 units								
No. 51	EHBF	189	226	217	216	212	179	213	216	246	254	276	302	298	274
♂	HV	113.0	106.5	112.0	117.4	112.2	112.2	92.2	81.9	77.3	69.7	72.4	68.6	67.5	65.1
17.2 kg	A	88.9	82.7	82.1	88.3	85.5	80.2	69.1	59.4	52.3	42.6	45.3	42.8	42.8	41.5
	HV-A	24.1	23.8	29.9	29.1	26.7	32.0	23.1	22.5	25.0	27.1	27.1	25.8	24.7	23.6
	HGO	45.5	53.9	65.1	63.0	56.9	57.3	48.2	48.6	61.5	68.8	74.7	78.0	73.5	64.3
	Insulin						←5 units								
Mean	EHBF		309	328	324	315	340	344	355	361	344	318	342		
15.8 kg	HV		89.8	89.2	88.4	89.4	83.1	70.6	58.6	55.1	61.3	64.2	65.6		
	A		75.1	73.9	73.6	74.6	65.2	54.6	44.4	38.5	39.3	40.3	40.8		
	HV-A		14.7	15.3	14.8	14.8	17.9	16.0	14.2	16.6	22.0	23.9	24.8		
	HGO		40.4	44.9	45.0	42.2	54.6	52.0	48.4	56.4	78.1	75.7	88.2		
	Insulin						←6.5 units								

* See Table I for abbreviations.

starting the insulin infusion, hepatic glucose output and HV-A glucose difference averaged 28.5 mg per minute and 6.7 mg per 100 ml, a fall of 63 and 73 per cent, respectively, from control values (Figures 2 and 4).

The administration of glucose following the start of the insulin infusion resulted in the greatest drop in hepatic glucose output although mean arterial glucose concentration remained below control values (Table V, Figures 2 and 5). After 30 minutes of insulin infusion, before glucose was administered, mean hepatic glucose

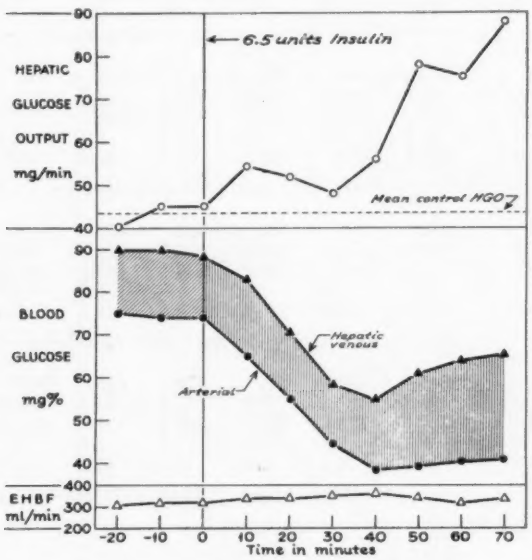

FIG. 3. MEAN CHANGES IN HEPATIC VENOUS AND ARTERIAL GLUCOSE CONCENTRATIONS, HEPATIC BLOOD FLOW, AND THE HEPATIC GLUCOSE OUTPUT AFTER THE RAPID INTRAVENOUS INJECTION OF GLUCAGON-FREE INSULIN.

output had fallen to 20.4 mg per minute, a 53 per cent decline from the control value of 43.3 mg per minute (Table V). During the last 30 minutes when glucose was infused with insulin, hepatic glucose output declined strikingly and averaged only 7.3 mg per minute, an 83 per cent reduction from the mean control. This marked depression in mean hepatic glucose output occurred despite a rise of only 5.3 mg per 100 ml in mean arterial glucose concentration, which remained 10.3 per cent below the mean control value (Figure 5). Not only was mean hepatic glucose output reduced to lower levels than in any of the

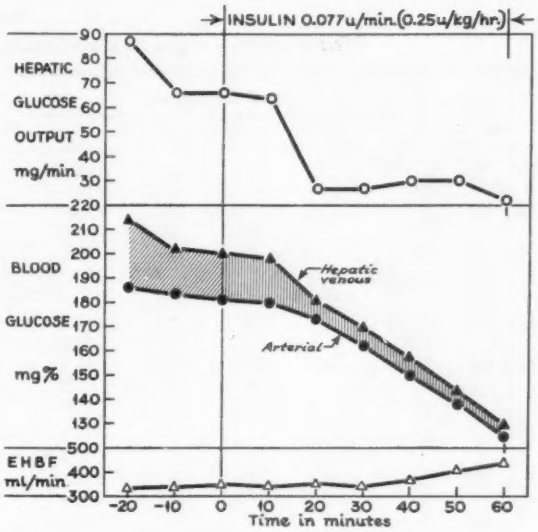

FIG. 4. MEAN CHANGES IN HEPATIC VENOUS AND ARTERIAL GLUCOSE CONCENTRATIONS, HEPATIC BLOOD FLOW, AND THE HEPATIC GLUCOSE OUTPUT IN DIABETIC DOGS DURING THE SLOW INTRAVENOUS INFUSION OF GLUCAGON-FREE INSULIN.

other experiments (Figure 2), but in Dog 119 (Table V) actual storage of glucose by the liver occurred when arterial blood glucose concentration was about 10 mg per 100 ml below the control level.

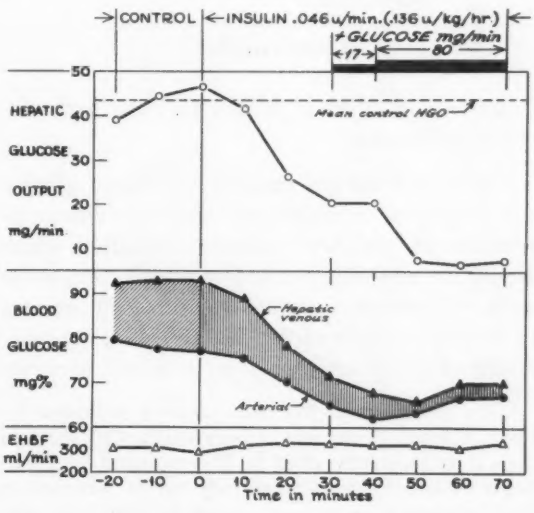

FIG. 5. MEAN CHANGES IN HEPATIC VENOUS AND ARTERIAL GLUCOSE CONCENTRATIONS, HEPATIC BLOOD FLOW, AND THE HEPATIC GLUCOSE OUTPUT DURING THE SLOW INFUSION OF GLUCAGON-FREE INSULIN PLUS GLUCOSE.

TABLE IV

Effect of infusion of glucagon-free insulin on the hepatic glucose output of diabetic dogs

Dog		Control values*					Time during insulin infusion					
		−30	−20	−10	0	Mean control	10	20	30	40	50	60
No. 42	EHBF		572	533	612	572	571	527	526	527	505	597
♂	HV		145.5	150.2	152.0	149.2	154.9	146.5	141.5	129.0	114.3	100.9
23.7 kg	A		134.5	128.7	136.3	133.1	140.2	138.7	130.3	119.8	105.4	94.1
	HV-A		11.0	21.5	15.7	16.1	14.7	7.8	11.2	9.2	8.9	8.8
	HGO		62.9	114.5	96.1	91.2	83.9	41.1	58.9	48.5	44.9	40.6
	Insulin						← 0.079 *units/min* (0.2 *units/kg/hr*) →					
No. 417	EHBF		181	175	164	173	185	183	169	178	189	193
♂	HV		215.0	199.2	193.6	202.6	177.3	166.5	158.4	150.4	143.1	132.0
17.2 kg	A		188.9	183.6	176.1	182.9	168.3	160.5	151.2	146.6	138.3	128.5
	HV-A		26.1	15.6	17.5	19.7	9.0	6.0	7.2	3.8	4.8	3.5
	HGO		47.2	27.4	28.9	34.5	16.7	11.0	12.2	6.8	9.1	6.8
	Insulin						← 0.086 *units/min* (0.3 *units/kg/hr*) →					
No. 529	EHBF	235	308	333	380	314	342	448	384	411	498	466
♀	HV	297.3	299.3	262.8	259.0	279.6	269.8	238.0	225.0	220.3	207.0	192.0
17.7 kg	A	235.0	237.5	242.5	238.3	238.3	239.8	233.8	220.0	209.0	198.5	187.5
	HV-A	62.3	61.8	20.3	20.7	41.3	30.0	4.2	5.0	11.3	8.5	4.5
	HGO	146.4	190.3	67.6	78.7	120.8	102.6	18.8	19.2	46.4	42.3	21.0
	Insulin						← 0.074 *units/min* (0.25 *units/kg/hr*) →					
No. 729	EHBF	267	283	317	246	277	282	264	289	402	473	531
♂	HV	212.7	198.2	196.2	198.4	201.4	190.4	174.4	155.1	131.6	114.1	95.1
16.8 kg	A	181.0	181.8	179.5	174.6	179.2	171.9	161.2	149.3	126.8	108.2	91.8
	HV-A	31.7	16.4	16.7	23.8	22.2	18.5	13.2	5.8	4.8	5.9	3.3
	HGO	84.6	46.4	52.9	58.5	60.6	52.2	34.8	16.8	19.3	27.9	17.5
	Insulin						← 0.070 *units/min* (0.25 *units/kg/hr*) →					
Mean	EHBF		336	339	350	334	345	356	342	379	416	447
	HV		214.5	202.1	200.7	208.2	198.1	181.4	170.0	157.8	144.6	130.0
18.9 kg	A		185.7	183.5	181.3	183.4	180.0	173.6	162.7	150.5	137.6	125.5
	HV-A		28.8	18.5	19.4	24.8	18.1	7.8	7.3	7.3	7.0	4.5
	HGO		86.7	65.6	65.6	76.8	63.9	26.4	26.8	30.2	31.0	21.5
	Insulin						← 0.077 *units/min* (0.25 *units/kg/hr*) →					

* See Table I for abbreviations.

2. *Calculated effect of insulin on peripheral glucose utilization*

These data on the changes in hepatic glucose output give some insight into the *approximate* alterations in peripheral glucose utilization which attend the slow infusion and rapid injection of insulin. Assuming a glucose space [3] of 30 per cent of the body weight of the dog (39–41), the magnitude of the glucose pool prior to and after insulin administration can be calculated. Since the hepatic contribution to this change in glucose pool is known, alterations in peripheral glucose utilization occurring simultaneously can be approximately estimated. The calculated data indicate three different types of response to insulin, apparently dependent upon the rate of insulin administration and the availability of glucose for peripheral utilization (Table VI). *First,* when insulin was administered by slow infusion in nondiabetic dogs, the decrease in mean hepatic glucose output accounted for the entire reduction in the size of the glucose pool, there being, therefore, no evidence of increased peripheral glucose utilization. *Second,* precisely the reverse changes occurred following the rapid injection of insulin. Since hepatic glucose output increased, the entire

[3] The glucose space has been variously estimated between 19.5 and 30 per cent of body weight (39, 40). In dogs it is apparently close to 25 per cent (41). The larger estimate, 30 per cent of body weight, was used in these calculations in order to correct for the 12 per cent higher concentration of glucose in plasma compared to whole blood and, therefore, to prevent overestimation of the hepatic contribution to total decrease in glucose pool.

TABLE V

Effect of the slow infusion of insulin and insulin plus glucose on the hepatic glucose output

Dog		Control values*				Time during insulin infusion									
		−20	−10	0	Mean control	10	20	30	40	50	60	70	80	90	100
No. 1222	EHBF	373	363	333	356	317	334	347	313	327	326	381	371	337	343
♂	HV	102.7	98.7	101.3	100.9	97.6	79.7	67.4	62.3	54.6	55.5	52.6	55.5	62.0	65.2
25.0 kg	A	93.6	90.2	86.5	90.1	82.8	71.4	62.3	56.2	52.1	51.5	50.9	52.6	60.3	62.3
	HV-A	9.1	8.5	14.8	10.8	14.8	8.3	5.1	6.1	2.5	4.0	1.7	2.9	1.7	2.9
	HGO	33.9	30.9	49.3	38.0	46.9	27.7	17.7	19.1	8.2	13.0	6.5	10.8	5.7	9.9
	Insulin					← 0.063 *units/min* →									
	Glucose									← 100 *mg/min* →			← 200 *mg/min* →		
No. 1215	EHBF	203	193	193	196	232	200	266	222	249	207	200	224	268	
♂	HV	97.1	101.6	106.7	101.8	102.2	86.3	77.8	80.6	76.1	73.0	70.7	77.5	78.1	
18.2 kg	A	80.6	80.1	82.3	81.0	83.5	77.2	69.3	72.1	69.8	66.1	66.7	72.4	73.5	
	HV-A	16.5	21.5	24.4	20.8	18.7	9.1	8.5	8.5	6.3	6.9	4.0	5.1	4.6	
	HGO	33.5	41.5	47.1	40.7	43.4	18.2	22.6	18.9	15.7	14.3	8.0	11.4	12.3	
	Insulin					← 0.05 *units/min* →									
	Glucose								← 75 *mg/min* →			← 150 *mg/min* →			
No. 1219	EHBF	166	164	193	174	268	283	214	253	248	275	236			
♀	HV	100.4	104.7	100.7	101.9	93.6	92.2	85.9	85.5	85.6	93.3	100.7			
18.0 kg	A	85.9	84.5	84.2	84.9	86.2	84.2	81.7	80.0	83.6	92.5	99.6			
	HV-A	14.5	20.2	16.5	17.0	7.4	8.0	4.2	5.5	2.0	0.8	1.1			
	HGO	24.1	33.1	31.8	29.7	19.8	19.0	9.0	13.9	5.0	2.2	2.6			
	Insulin					← 0.045 *units/min* →									
	Glucose									← 100 *mg/min* →					
No. 1559	EHBF	290	332	308	310	307	334	267	236	230	245	267			
♂	HV	56.0	52.0	52.6	53.5	44.0	39.1	34.8	32.3	44.6	57.1	60.3			
15.4 kg	A	43.1	43.4	41.1	42.5	36.0	30.3	29.1	26.0	40.3	54.3	58.3			
	HV-A	12.9	8.6	11.5	11.0	8.0	8.8	5.7	6.3	4.3	2.8	2.0			
	HGO	37.4	28.6	35.4	33.8	24.5	29.3	15.2	14.9	9.9	6.9	5.3			
	Insulin					← 0.026 *units/min* →									
	Glucose									← 75 *mg/min* →					
No. 1118	EHBF	283	285	245	271	264	261	287	284	286	268	299	297	306	
♀	HV	99.3	104.1	104.1	102.5	104.1	88.4	85.7	76.4	71.1	66.6	60.9	56.1	75.9	
20.0 kg	A	86.0	84.9	83.2	84.7	86.0	77.8	74.2	70.8	65.7	60.9	55.3	45.0	46.2	
	HV-A	13.3	19.2	20.9	17.8	18.1	10.6	11.5	5.6	5.4	5.7	5.6	11.1	29.7	
	HGO	37.6	54.7	51.2	47.8	47.8	27.7	30.3	15.9	15.4	15.3	16.7	33.0	90.9	
	Insulin					← 0.04 *units/min* →			← 0.065 *units/min* →				None →		
	Glucose								← 25 *mg/min* →				None →		

* See Table I for abbreviations.

TABLE V—*Continued*

Dog		Control values*			Mean control	Time during insulin infusion									
		−20	−10	0		10	20	30	40	50	60	70	80	90	100
No. 119	EHBF	521	512	497	510	551	592	636	606	609	533	583			
♂	HV	99.0	96.2	94.0	96.3	92.5	87.1	78.8	74.0	68.5	72.0	73.7			
25.5 kg	A	86.3	81.1	81.1	82.8	80.3	80.8	74.5	67.1	69.7	74.3	72.8			
	HV-A	12.7	15.1	12.9	13.6	12.2	6.3	4.3	6.9	+1.2	+2.3	0.9			
	HGO	66.7	77.3	64.1	69.4	67.2	37.3	27.4	40.0	+7.3	+12.3	5.3			
	Insulin					← 0.04 *units/min* →									
	Glucose									← 100 *mg/min* →					
Mean	EHBF	306	308	295	302	323	334	336	319	325	309	328			
20.3 kg	HV	92.3	92.9	93.2	92.7	89.0	78.8	71.7	68.5	66.7	69.5	69.8			
	A	79.3	77.4	76.4	77.6	75.8	70.3	65.2	62.0	63.5	66.6	67.3			
	HV-A	13.0	15.5	16.4	15.1	13.2	8.5	6.5	6.5	3.2	3.0	2.5			
	HGO	38.7	44.4	46.5	43.3	41.6	26.5	20.4	20.5	7.3	6.6	7.4			
	Insulin					← 0.046 *units/min* (0.136 *units/kg/hr*) →									
	Glucose								← 80 *mg/min* →						

TABLE VI

Contribution of the change in hepatic glucose output and the calculated change in peripheral glucose utilization to the decrease in the glucose pool after insulin administration

Experiment	Slow infusion insulin	Rapid injection insulin	Slow infusion insulin diabetic dogs	Infusion insulin plus glucose
Mean weight/kg	16	15.8	18.9	20.3
Glucose space/L	16 × 0.3 = 4.8	15.8 × 0.3 = 4.74	18.9 × 0.3 = 5.67	20.3 × 0.3 = 6.09
Glucose pool/mg				
Initial size	79.8 *mg per 100 ml* × 48 = 3,830	74.6 *mg per 100 ml* × 47.4 = 3,536	183.4 *mg per 100 ml* × 56.7 = 10,400	77.6 *mg per 100 ml* × 60.9 = 4,725
Final size	59.0 *mg per 100 ml* × 48 = 2,830	38.5 *mg per 100 ml* × 47.4 = 1,825	125.5 *mg per 100 ml* × 56.7 = 7,116	67.3 *mg per 100 ml* × 60.9 = 4,100
Change	998	1,711	3,284	625
				(Added to pool by infusion) 2,570
Hepatic glucose output (HGO)/*mg/min*				
Mean control	45.0	42.2	76.8	43.3
Mean insulin	25.3	51.1†	37.9	21.3
Change	↓*19.7	↑ 8.9	↓38.9	↓22.0
Change in glucose pool attributable to change in HGO/mg	19.7 × 60 min = ↓1,182	8.9 × 40 min = ↑356	38.9 × 60 min = ↓2,334	22 × 70 min = ↓1,514
Calculated change in peripheral glucose utilization	(998 − 1,182) ↓184 *mg/60 min* ↓3 *mg/min*	(1,711 + 356) ↑2,067 *mg/40 min* ↑51 *mg/min*	(3,284 − 2,334) ↑950 *mg/60 min* ↑15.8 *mg/min*	[625 + 2,570] − 1,514 ↑1,681 *mg/70 min* ↑24.0 *mg/min*
Per cent change in glucose pool attributable to:				
Decrease in HGO	100	0	71	47.4
Increase in peripheral glucose utilization	0	100	29	52.6

* ↓ Denotes decrease; ↑ denotes increase.

† Mean HGO during the time arterial glucose concentration was falling.

decline in the glucose pool must have been the consequence of a marked increase in peripheral glucose utilization. These data confirm a previous study from this laboratory (14) in which the effects of the slow infusion and rapid injection of insulin on peripheral glucose utilization, as reflected by changes in femoral arteriovenous glucose difference, were compared in normal human subjects. Following the rapid injection of insulin, mean arteriovenous glucose difference increased from 1.8 to 9.3 mg per 100 ml, whereas during the fall in arterial glucose concentration which attended the slow infusion of insulin, mean arteriovenous glucose difference narrowed from 1.6 to 0.5 mg per 100 ml. *Third,* when glucose was delivered to the peripheral tissues, as in the diabetic dogs and in those dogs given glucose plus insulin, the decrease in the glucose pool after insulin was the consequence of *both* a decline in the hepatic output of glucose and of an increase in peripheral glucose utilization (Table VI).

3. *The effect of the establishment of a portacaval shunt on hepatic blood flow and hepatic glucose metabolism*

In the present studies control hepatic blood flow in dogs with portacaval shunts averaged 281 ml per minute or 15 ± 2.8 ml per kg per minute. In 17 other studies in dogs without portacaval shunts, which were performed in this laboratory utilizing the same technics, hepatic blood flow averaged 38.3 ml per kg per minute, a value similar to that reported by others (42–44).

Despite the large decrement in hepatic blood flow, there was no apparent change in hepatic glucose output following the establishment of the portacaval shunt either in these or in other studies (45). Mean control hepatic glucose output of 2.36 ± 0.9 mg per kg per minute found in these studies compares closely with values of 2.0 ± 0.2 mg per kg per minute reported by Lipscomb and Crandall (46) and 2.29 mg per kg per minute recalculated from the data of Steele and associates (47).

DISCUSSION

Under conditions of these experiments, an immediate and significant decline in hepatic glucose output occurred in response to insulin whenever it was administered in a manner calculated to reduce counter-regulatory mechanisms to hypoglycemia (Tables I, IV and V). By contrast, when insulin was given by rapid intravenous injection, either no change or an increase in hepatic glucose output was found (Table III). This marked difference in the hepatic action of insulin following the different rates of insulin administration is probably related to the magnitude of stimulation of counter-regulatory mechanisms to hypoglycemia. During the slow infusion of insulin, mean arterial glucose concentration fell only 21 mg per 100 ml in 60 minutes, whereas following the rapid injection of insulin, mean arterial glucose concentration dropped to 38.5 mg per 100 ml in only 40 minutes. The greater release of epinephrine, adrenal cortical hormones and glucagon which would be anticipated when profound hypoglycemia supervenes (21–30) apparently counterbalanced and overwhelmed the effect of insulin on the liver.

The greater decline in hepatic glucose output which occurred when the duration and magnitude of hypoglycemia was further reduced by administering glucose after insulin infusion had been started (Table V), lends strong support to the thesis that by diminishing the homeostatic response to hypoglycemia, a more potent hepatic action of insulin can be unmasked. When insulin alone was infused mean hepatic glucose output fell 56 per cent during the last 30 minutes of infusion, whereas when glucose was added mean hepatic glucose output fell 83 per cent during a similar period of time (Figure 2).

In the experiments in which a direct hepatic effect was demonstrated, insulin was infused into a peripheral vein and reached the liver only after dilution in the blood volume and in the proportion of hepatic blood flow to total cardiac output. This would suggest that even smaller amounts of insulin injected into the portal vein would have a similar, if not greater, hepatic effect. Other published data from this laboratory support the contention that intraportally administered insulin has a greater hepatic action than has insulin given via a peripheral vein (13).

The calculated decline in the glucose pool could be ascribed in large part, if not entirely, to the decrease in hepatic glucose output when insulin alone was administered by slow infusion and to

the increase in peripheral glucose utilization following the rapid injection of insulin. Just as the hepatic effect of insulin was masked by counterregulatory mechanisms to hypoglycemia, so too was the peripheral effect obscured by the decreased delivery of glucose to the peripheral tissues during insulin infusion (Table VI). In a sense, both circumstances are unphysiological since insulin secretion is usually stimulated by the rising blood glucose concentration that follows a carbohydrate load. Under physiological conditions the magnitude of the hepatic and peripheral contributions to the decrease in the glucose pool more likely approaches that found in the diabetic dogs and in the dogs given insulin plus glucose than that which followed either the slow infusion alone or the rapid injection of insulin (Table VI).

The results of these experiments, which show by direct measurement an immediate effect of insulin upon hepatic glucose output, differ from many other *in vivo* and *in vitro* studies which have failed to elicit a consistent and reproducible effect of insulin on the liver (5–12). While a positive *in vitro* effect of insulin on hepatic glucose metabolism is probably meaningful, the failure to find a consistent effect, or any effect, does not constitute unequivocal evidence that such an action does not exist in the intact organism. The failure to obtain an *in vitro* effect may be linked with the difficulty in maintaining the integrity of both morphologic and functional organization of the liver and with the inability to simulate precisely *in vivo* nutritional conditions. Stetten has recently aired the problems inherent in equating rates of metabolic processes or even their presence from *in vitro* studies on isolated tissues with that which pertains in intact organisms, especially insofar as the liver is concerned (48). The rate-limiting step of a metabolic process in a liver slice may be the rate of transfer of nutrient from the bath to the "liver slice sloshing leisurely in a vessel" (48), a condition quite dissimilar from *in vivo* studies in which each hepatic cell is in intimate contact with the perfusing blood.

Other *in vivo* studies have produced inferential data indicating that insulin has a profound and significant effect on hepatic glucose output (16–19, 49–53), a minor and physiologically insignificant effect (7), and no effect (6, 8–11). In view of these different results, a critical analysis of the various technics used is pertinent to help resolve the apparent paradox of these conflicting data. The following differences in experimental design may be related to these discrepant results. *1*) In some studies changes in splanchnic rather than in hepatic glucose output were measured (49–52); *2*) in most studies insulin was administered in amounts and at rates that evoked severe arterial hypoglycemia (6–11, 17, 50, 51) which, in the light of the data from the present study, probably precludes the demonstration of a hepatic effect of insulin; *3*) isotopic technics, which measure only hepatic glucose production and are incapable of measuring hepatic glucose utilization, were used (6–11, 17, 18).

Although Bondy, Bloom, Whitner and Farrar (49), and Bearn, Billing and Sherlock (50, 51) reported an immediate effect of insulin on splanchnic glucose metabolism, the limitations of the hepatic venous catheter technic insofar as the measurement of *hepatic* in contrast to *splanchnic* glucose metabolism in intact animals is concerned, have been pointed out by Bondy (49) and others (52, 53). Shoemaker, Mahler and Ashmore (11) attempted to separate hepatic from extrahepatic splanchnic glucose metabolism by measuring both splanchnic blood flow and the concentrations of glucose in arterial, portal and hepatic venous blood. No decrease in hepatic glucose output following insulin was observed, a finding similar to those experiments in the present study in which marked arterial hypoglycemia was produced; in all but two of their experiments, insulin was administered at rates and in amounts which evoked severe arterial hypoglycemia. Their failure to find a decrease in hepatic glucose output in the two other experiments in which a gradual decline in blood glucose concentration was produced by slow insulin infusion, may be related to their inability to completely separate the hepatic and extrahepatic splanchnic beds by assuming that 80 per cent of total hepatic blood flow is continuously derived from the portal venous inflow. Such an assumption may be unwarranted in view of the wide and momentary fluctuation in the portal venous contribution to total hepatic blood flow reported by Soskin, Essex, Herrick and Mann (20).

Two other types of studies have been designed to measure the effect of insulin upon hepatic glucose metabolism by following changes in the specific activity of blood glucose, labeled by the administration of a tracer dose of uniformly labeled glucose-C^{14}. Dunn and associates (17) and Jacobs and co-workers (18), utilizing the "single injection" technic, reported a significant decline in hepatic glucose output after insulin administration, particularly when severe hypoglycemia was avoided, whereas no significant hepatic effect of insulin was obtained with the "primer-infusion" technic (7–9). Proponents of the "primer-infusion" technic (8–9) have leveled serious criticism against the interpretation of data from studies using the "single injection" method. The "plateauing" effect following insulin administration in the "single injection" technic, is claimed to be the consequence of a diminished hepatic output of unlabeled glucose (17, 18). Others have considered it, in whole or in part, to be an artifact (6, 8, 9, 11, 12) and attributable to recycling of labeled intermediaries, or to the release of glucose with high isotopic abundance not only from the outer tiers of hepatic glycogen labeled during initial equilibration (8, 12) but also from the mucosal cells of the gastrointestinal tract (11). Moreover, in the "single injection" technic the exponential decline in specific activity may represent hepatic output of C^{12}-glucose during a steady state but not necessarily during rapid changes in glucose pool size when the rate of change in pool size is greater than the rate of mixing throughout compartments of the pool (6–9, 54). To over-

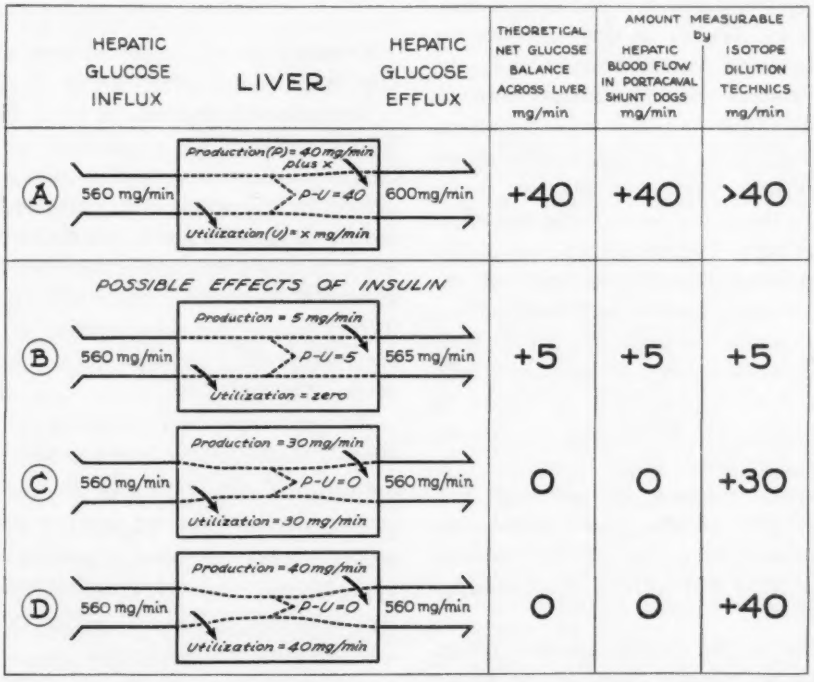

FIG. 6. COMPARISON OF THE THEORETICAL CHANGES IN THE NET BALANCE OF GLUCOSE ACROSS THE LIVER WITH THOSE MEASURED BY THE HEPATIC BLOOD FLOW TECHNIC IN DOGS WITH PORTACAVAL SHUNTS AND BY THE ISOTOPE (GLUCOSE-C^{14}) DILUTION TECHNICS AFTER INSULIN ADMINISTRATION. The label A refers to the conditions in the postabsorptive state prior to insulin administration. The labels B, C and D identify the theoretically possible effects of insulin on the liver. The magnitude of the theoretical change in net glucose balance across the liver is compared with the magnitude of change each technic is capable of quantitating. Hepatic glucose influx is the total amount of glucose brought to the liver; hepatic efflux the total amount leaving the liver. Efflux equals influx plus hepatic production minus hepatic utilization. See text for details.

come these objections, Tarding and Schambye combined measurement of total splanchnic glucose output with that of the specific activity of glucose entering and leaving the liver (6), thereby permitting quantitation of hepatic release of unlabeled glucose even at times of rapid change in pool size. Such studies also failed to demonstrate a hepatic action of insulin (6).

In both Tarding's study and in those using the "primer-infusion" technic (7–9), insulin was administered by rapid intravenous injection in most experiments. However, in those instances in which published data are available and in which insulin was given by slow infusion (6, 9), no significant decrease in hepatic glucose output was found, a result different from that reported in the present studies.

A serious objection, in the opinion of the present authors, to all the aforementioned isotope dilution studies (6–11, 17–19) is that such studies can measure only *new glucose production* by the liver. *Hepatic glucose utilization cannot be measured* nor can it be differentiated from peripheral glucose utilization. Only if insulin exclusively or mainly affected new glucose production would these be valid methods for determining the effect of insulin on the liver (Figure 6, Parts A and B). If, on the other hand, initially the major or exclusive hepatic effect of insulin was the stimulation of glucose utilization by the liver cells, the methods would not be sensitive enough to measure a relatively small change in production or would be valueless as a technic for determining the hepatic effect of insulin (Figure 6, Parts C and D). In view of these considerations, the failure to find an effect of insulin on hepatic glucose metabolism by isotopic dilution technics (6–11) does not constitute definitive proof that such an effect does not occur.

The similarity in the hepatic glucose output (2.29 mg per kg per minute) found in the isotope dilution studies (47) which measure hepatic glucose production, and in the present studies (2.36 mg per kg per minute) which measure the net balance of glucose across the liver (production minus utilization), suggests that in the postabsorptive state, prior to insulin administration, hepatic utilization of glucose, like that of muscle (55), is small. Failure of isotope dilution technics (6–9) to detect any decrease in hepatic glucose production after insulin administration, whereas an immediate effect was noted in the present studies, implies that initially the major hepatic effect of insulin is the stimulation of glucose utilization by the liver cells, an action quite similar to that which insulin evokes in muscle. In view of the free permeability of liver to glucose (56) in contrast to the permeability of muscle to glucose (57, 58), it is possible that in the liver insulin acts either by altering the permeability of some intracellular membrane to glucose, simulating its action on muscle cell membrane, or by changing the activity of hepatic glucokinase. The rapidity with which the change in hepatic glucose output occurred both in normal and diabetic dogs in the present studies may favor its action on an intracellular membrane.

SUMMARY AND CONCLUSIONS

Twenty-four experiments were performed on dogs with complete end-to-side portacaval shunts to determine whether insulin has a direct effect on hepatic carbohydrate metabolism and also to ascertain whether the rate of insulin administration altered the magnitude of its peripheral and hepatic action. Dogs with portacaval shunts were selected since, in this preparation, the liver is completely separated from the extrahepatic splanchnic bed, thereby permitting measurement of glucose balance across the liver alone rather than across the entire splanchnic bed.

An immediate and physiologically significant effect of insulin on the liver has been demonstrated for the first time by direct measurement; when insulin was administered by slow intravenous infusion in a manner which minimized or prevented hypoglycemia and its attendant counter-regulatory response, a prompt decline in hepatic glucose output of considerable magnitude ensued. In contrast, when insulin was administered by rapid intravenous injection, hepatic glucose output either remained unchanged or increased. The failure of other studies to find a hepatic effect of insulin may be related to the rapid rate of insulin administration and also to the fact that the isotopic dilution technics permit the measurement only of hepatic glucose production and cannot quantitate hepatic glucose utilization.

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THE METABOLIC EFFECTS OF THE ACETIC AND PROPIONIC ACID ANALOGS OF THYROXINE AND TRIIODOTHYRONINE *

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Within the last six years the identification of several new substances with thyroid hormone-like activity has led to a re-evaluation of the physiological role of thyroxine and of its analogs and derivatives. Since its isolation in 1915 (1) thyroxine was considered the most biologically potent of all simple thyroactive materials until the discovery by Gross and Pitt-Rivers (2) and by Roche, Lissitzky and Michel (3) that 3,5,3′-triiodo-L-thyronine (trit) was four to five times more effective in both animals and man.

The acetic acid analogs of thyroxine and trit have been actively investigated since 1953 (4). Thibault and Pitt-Rivers (5–7) described a unique action of these compounds in that they produced an increased oxygen consumption *in vitro* without previous injection into animals. The maximum effects were reached in 15 minutes and disappeared after 90 minutes. A single injection of these drugs into thyroidectomized rats increased oxygen consumption within two hours. Although these studies have not been confirmed (8, 9), they resulted in the postulate that this might be the form of the thyroid hormone utilized at the tissue level. This suggestion became even more attractive when these substances were isolated from tissues and homogenates (10–17).

Other studies in man have suggested a dissociation of responses to those drugs (18–25). In patients with hypothyroidism, (19, 21) it was noted that on small doses of the acetic acid analogs the serum cholesterol level fell, the nitrogen and phosphorus balances became negative, and the patients were clinically improved without any significant change in basal metabolic rate. Other studies in euthyroid subjects have demonstrated a lowering of the serum cholesterol level, which was usually temporary, with no effect on the basal metabolic rate, following the administration of small amounts of these acetic acid analogs (18, 20–22, 24–27). These findings suggested that there might be more than one thyroid hormone, that the acetic acid derivatives were primarily effective in cholesterol metabolism, or that a change in the basal metabolic rate was not an obligatory accompaniment of thyroid hormone activity. Other workers, (9, 26–29) have been unable to find a unique dissociation of responses to these compounds in patients with myxedema, although one study (29) has demonstrated a dose-related dissociation of responses to all thyroactive substances investigated.

The present studies were designed to evaluate further, both in animals and in man, the metabolic actions of 3,5,3′-triiodothyroacetic acid (triac), 3,5,3′,5′-tetraiodothyroacetic acid (tetrac), 3,5,3′-triiodothyropropionic acid (triprop), and 3,5,3′,5′-tetraiodothyropropionic acid (tetprop). The effects of these compounds were compared qualitatively and quantitatively with those of L-thyroxine and trit.

MATERIALS AND METHODS

Animal studies. Thyroidectomized rats were used as sensitive test animals on which to determine the comparative metabolic effects of L-thyroxine, trit, and their respective acetic acid and propionic acid analogs.[1] Four

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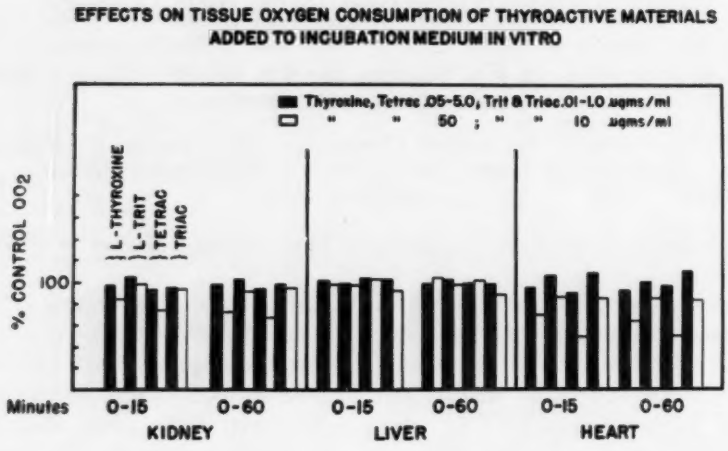

FIG. 1. OXYGEN CONSUMPTION OF SLICES OF KIDNEY, LIVER AND HEART FROM THYROIDECTOMIZED RATS. Studies were made in Krebs-Ringer phosphate glucose solution to which were added the quantities shown of thyroxine, tetrac, trit or triac. Values are expressed as per cent of the control flasks containing the same amount of alkali (0.005 N, final concentration) as that used to dissolve the thyroactive materials. Each bar is the average of eight experiments with readings covering the first 15 minutes and first 60 minutes after a preliminary 15 minute equilibrium period at 37° C.

types of studies were made: *a*) the *in vitro* response of kidney, liver and heart slices to the thyroactive materials; *b*) the response of heart, diaphragm and skeletal muscle oxygen consumption to single large doses of the thyroactive materials injected into thyroidectomized rats; *c*) the action of similar single large doses of these compounds on the basal metabolic rates of thyroidectomized rats; and *d*) the effects of repeated subcutaneous injections of the thyroactive substances on the metabolic rate of seven selected tissues of thyroidectomized rats. In every case the animals were etherized, exsanguinated and the desired tissues removed. These were kept moist with Ringer's solution at 5° C while making suitable preparations (blunt-dissected muscle fiber bundles, isolation of portions of diaphragm and razor blade slices of other tissues) for study of their oxygen consumption. These measurements were made with the standard Barcroft-Warburg technic in a phosphate-buffered Krebs-Ringer solution with glucose as the substrate.

Clinical studies. Clinical effects of these compounds were evaluated in three patients with primary hypothyroidism who were studied while being treated with one or more of the following compounds: trit, triac, tetrac, triprop, tetprop, thyroxine or desiccated thyroid substance.

All patients were studied on the metabolic ward of Birmingham Veterans Administration Hospital. Basal metabolic rates (BMR) were determined by a member of the research group using a Benedict-Roth machine. Measurements were made at 8:00 a.m. prior to the patients' daily medication. Pentobarbital, 90 mg, was routinely given orally at bed time the night before the measurement was made. The serum protein-bound iodine levels (PBI) were determined by a modification of the Barker Humphrey and Soley alkaline-ash method (30). The serum cholesterol levels were determined by the method of Pearson, Stern and McGavack (31). Thyroxine and dessicated thyroid were administered orally in a single morning dose. Trit, triac, tetrac, triprop and tetprop were administered orally in two or three divided daily doses. Measurements of the BMR, serum cholesterol and serum protein-bound iodine levels were made at one to three day intervals. In general, medication was held constant at each dose level until stabilization of these measurements had been obtained.

Clinical summaries. Case 1. F.S., a 62 year old male retired farmer, was admitted with typical primary myxedema. He was found to have a BMR of − 37 per cent, a serum cholesterol level of 452 mg per 100 ml, less than 1 per cent uptake of I^{131} over the thyroid at 24 hours, and a serum protein-bound iodine level of 1.7 µg per 100 ml. Forty-eight hours after administering 30 units of thyroid-stimulating hormone (TSH) intramuscularly, the 24 hour thyroidal I^{131} uptake was 6 per cent, which was interpreted as indicating primary hypothyroidism.

He was treated with increasing doses of trit until he gradually became euthyroid. Treatment was then withheld until he was again myxedematous. At this time therapy was instituted with tetrac which was gradu-

for some of the acetic acid analogs; and *d*) Dr. Michael C. Barry, Division of Clinical Research, The Warner-Lambert Research Institute, for the majority of the acetic and all of the propionic acid analogs.

ally increased until he was again euthyroid. He was then placed successively on triac, thyroxine, and dessicated thyroid. The doses of the last three compounds were adjusted so as to maintain clinical euthyroidism and normal basal metabolic rates.

Case 2. T.H., a 62 year old male construction worker, entered the hospital with signs and symptoms suggesting primary hypothyroidism. The BMR was −43 per cent. The serum protein-bound iodine level was 1.0 μg per 100 ml and the serum cholesterol was 386 mg per 100 ml. The I^{131} uptake over the thyroid was 8.9 per cent in 24 hours; two days after administering 20 units of TSH intramuscularly the thyroidal uptake of I^{131} was 7.6 per cent.

Therapy was started with small doses of triiodothyronine, which were gradually increased until he was clinically euthyroid and had normal basal metabolic rates. He was then placed on triac and tetrac successively, at dose levels which were adjusted to maintain clinical euthyroidism and normal basal metabolic rates. Therapy was then withdrawn until he was again myxedematous, after which he was treated with triprop in gradually increasing doses until euthyroidism was again established. He was then placed on thyroxine and desiccated thyroid in turn at dose levels adjusted to maintain clinical euthyroidism and normal basal metabolic rates.

Case 3. R.M., a 48 year old male carpenter, was admitted to the hospital with a history of hypothyroidism following a therapeutic dose of I^{131} given five months previously for toxic nodular goiter. His BMR was −38 per cent, the serum protein-bound iodine level was 3.1 μg per 100 ml, and the serum cholesterol level was 410 mg per 100 ml. The 24 hour thyroidal uptake of a tracer dose of I^{131} was 3.9 per cent. He was treated with tetprop in gradually increasing doses until clinical euthyroidism and normal basal metabolic rates were established.

OBSERVATIONS

Animal studies. Many unsuccessful attempts to demonstrate an acceleration of tissue oxygen consumption by the *in vitro* addition of thyroactive substances are summarized in Figure 1. Thyroxine and tetrac were without effect on slices of kidney, liver and heart from thyroidectomized animals when 0.05 to 5.0 μg were added per ml of incubation medium, as were trit and triac at dose levels of from 0.01 to 10.0 μg. At a level of 50 μg of thyroxine or tetrac per ml, depression of heart and kidney metabolism was evident.

To answer the possibility that the compounds applied *in vitro* above were not adequately penetrating the tissues, thyroidectomized animals were injected subcutaneously with one of the following: 2.0 mg thyroxine per kg body weight, 2.0 mg tetrac, 1.0 mg trit or 1.0 mg triac. After allow-

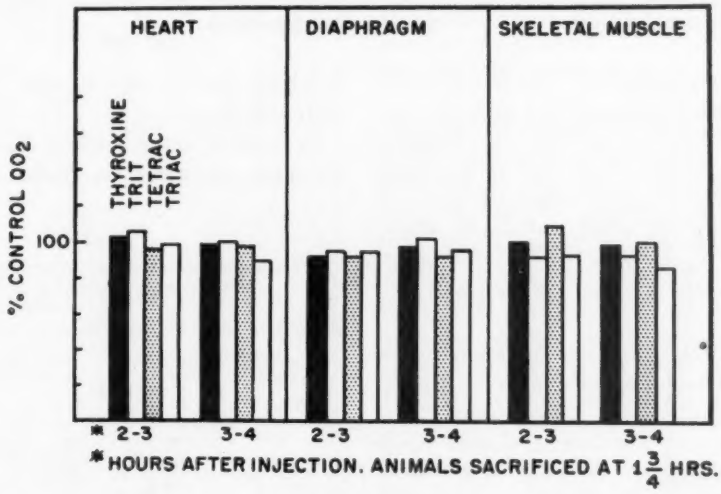

FIG. 2. THE OXYGEN CONSUMPTION OF HEART, DIAPHRAGM AND SKELETAL MUSCLE REMOVED FROM THYROIDECTOMIZED RATS AFTER A SINGLE SUBCUTANEOUS INJECTION OF THYROXINE (2 MG PER KG), TRIT (1 MG PER KG), TETRAC (2 MG PER KG) OR TRIAC (1 MG PER KG). Animals were sacrificed at 1.75 hours after injection and tissue metabolism was studied at 37° C from two to three and from three to four hours after the injection. Each value, the average of six experiments, is expressed as the per cent of a corresponding saline control-injected animal.

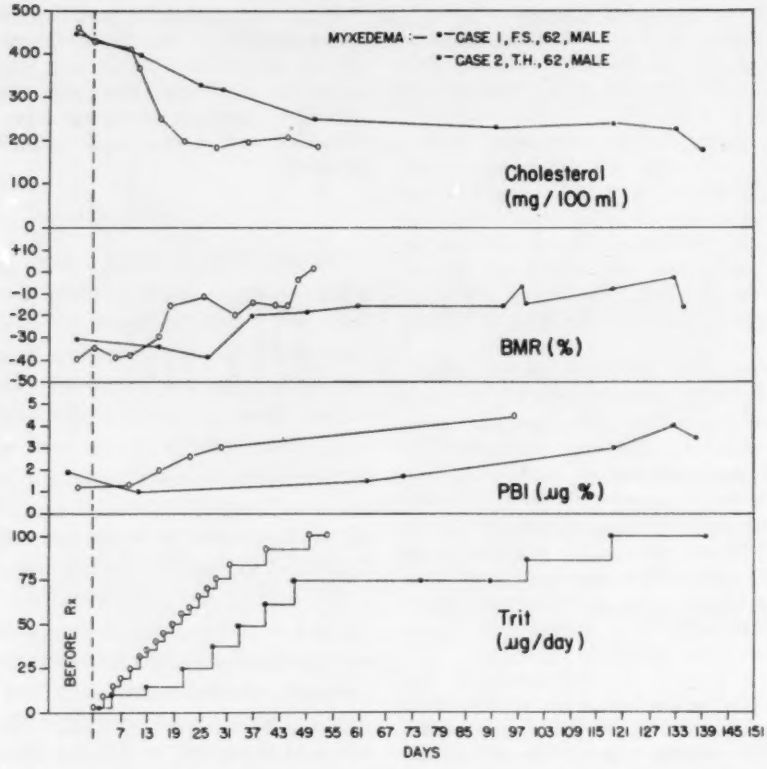

FIG. 3. THE EFFECT OF GRADUALLY INCREASING DOSES OF TRIT ON THE BASAL METABOLIC RATE, SERUM CHOLESTEROL LEVEL, AND SERUM PROTEIN-BOUND IODINE LEVEL OF TWO PATIENTS WITH PRIMARY HYPOTHYROIDISM.

ing 1.75 hours for absorption, the animals were sacrificed, and heart, diaphragm and skeletal muscle were prepared at 5° C for oxygen consumption studies. It is evident from Figure 2 that no consistent metabolic changes were produced in these tissues during the third and fourth hours after injection of the animals, the time of maximum effect previously reported for the acetic acid analogs (5).

Paralleling the lack of *in vitro* or immediate *in vivo* oxygen consumption effects just demonstrated was a similar series of whole-animal BMR measurements covering the four hour time range after a single subcutaneous injection of the same compounds. The results are shown in Table I, as percentage of BMR determinations obtained on the same uninjected thyroidectomized animals. There was a slight elevation in metabolic rate from 0.5 to 1.5 hours postinjection, probably because of the necessary handling of the animals. After that period, the BMR's settled down to the ± 10 per cent range usually encountered with such animals.

In contrast to the absence of *in vitro* or immediate *in vivo* oxygen consumption responses to these

TABLE I

Basal metabolic rates of thyroidectomized rats after subcutaneous injection of thyroxine, trit, tetrac or triac

Hours after injection	Thyroxine 2 mg/kg	Trit 1 mg/kg	Tetrac 2 mg/kg	Triac 1 mg/kg
Control*	80.6	82.3	79.4	77.0
	%	%	%	%
0.5–1.0	106.7	109.9	112.7	103.1
1.0–1.5	118.4	107.2	110.8	96.7
1.5–2.0	95.1	93.5	95.2	102.8
2.0–2.5	104.0	104.7	103.3	111.6
2.5–3.0	112.6	96.1	109.2	110.3
3.0–3.5	105.0	107.9	107.5	104.2
3.5–4.0	108.6	100.0	105.3	108.9

* Postabsorptive oxygen consumption is given as milliliters per 100 g body weight per hour prior to injection; all other figures are percentages of control. There were eight animals in each group.

thyroactive substances are results (Table II) showing that the compounds given to thyroidectomized animals in adequate doses over a more extended time are capable of causing appreciable increases in metabolic rate. These data were obtained by injecting subcutaneously the dose shown once each day for four days, then sacrificing the animals on the fifth day. The doses shown were selected because the increases in metabolic rates of the various tissues were all in the range of 25 to 35 per cent above controls. Computed on such a basis, both triac and triprop were 3.4 and 3.8 times as active as tetrac and tetprop, respectively, an enhancement approaching the 4.3 ratio of trit to thyroxine.

Clinical studies. Improvement in hypothyroidism was obtained from all substances studied when given in large enough doses. Cases 1 and 2 were treated initially with trit, starting with small

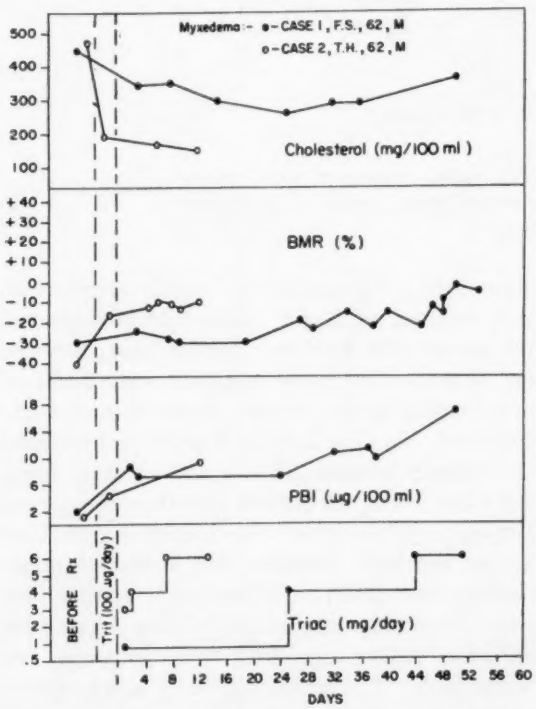

FIG. 4. THE EFFECT OF TRIAC ON THE BASAL METABOLIC RATE, SERUM CHOLESTEROL LEVEL, AND SERUM PROTEIN-BOUND IODINE LEVEL OF TWO PATIENTS WITH PRIMARY HYPOTHYROIDISM. Although Case 1 was myxedematous, the serum protein-bound iodine level remained elevated as a result of tetrac administration which had been withdrawn one month previously. Triac was given to Case 2 immediately after stopping trit.

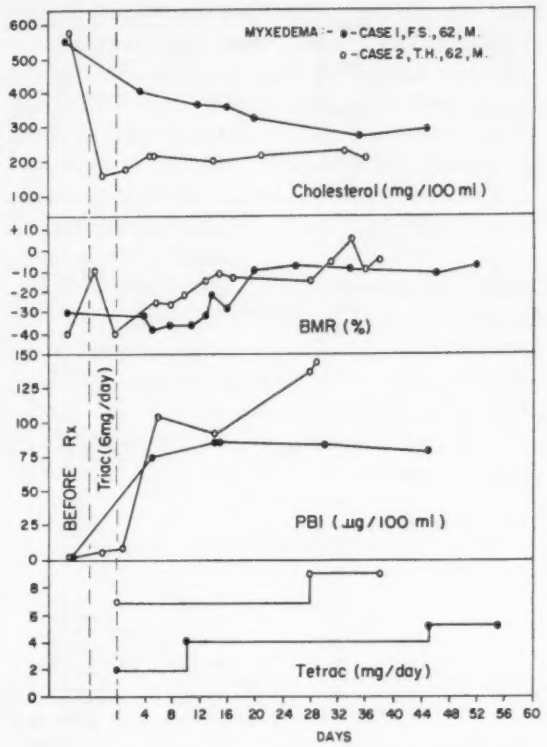

FIG. 5. THE EFFECT OF TETRAC ON THE BASAL METABOLIC RATE, SERUM CHOLESTEROL LEVEL, AND SERUM PROTEIN-BOUND IODINE LEVEL OF TWO PATIENTS WITH PRIMARY HYPOTHYROIDISM. Tetrac was given to Case 2 immediately after stopping triac.

oral doses and gradually increasing to a maximum maintenance dose of 100 µg per day. Increments of 5 µg were made every third day up to a dose of 80 µg in Case 2, and somewhat more slowly in Case 1 (Figure 3). The basal metabolic rate did not start to rise until a dose of approximately 40 µg was reached. In both cases, however, it was observed that the cholesterol was consistently reduced on doses of 20 to 30 µg per day (Figure 3). At this dose level clinical improvement was also manifest. Both patients were clinically slightly hyperthyroid on a dose of 100 µg per day and were euthyroid on 90 µg per day. The BMR returned to normal and the serum PBI level remained in the myxedematous range as expected (32–34).

As illustrated in Figure 4, triac was begun in Case 1 when the patient was myxedematous, with elevated serum cholesterol levels and low BMR's, but with a persistently elevated serum PBI level as

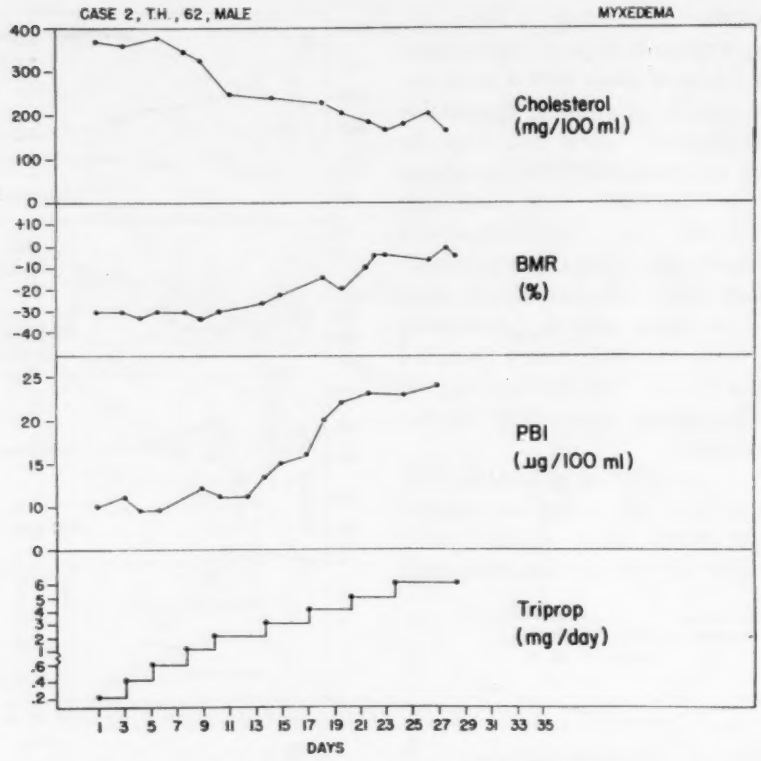

FIG. 6. THE EFFECT OF TRIPROP ON THE BASAL METABOLIC RATE, SERUM CHOLESTEROL LEVEL, AND SERUM PROTEIN-BOUND IODINE LEVEL OF A PATIENT WITH PRIMARY HYPOTHYROIDISM.

a result of tetrac administration which had been discontinued one month previously. In Case 2, triac was substituted for trit in doses which were adjusted to maintain euthyroidism. Triac was found to be weakly thyroactive, and 6 mg per day was required for maintenance of euthyroidism in both Cases 1 and 2. The response of Case 1 to triac again demonstrates a depression of the serum cholesterol level without a concomitant increase in BMR when small amounts of thyroactive substances were given. The serum PBI levels were variable, but were definitely elevated out of proportion to the metabolic state (Figure 4).

As illustrated in Figure 5, the administration of tetrac to Case 1 was begun when he was myxedematous; whereas in Case 2, tetrac was substituted for triac in doses adjusted to maintain euthyroidism. As with trit and triac, it was noted that the serum cholesterol level dropped significantly in Case 1 on small doses of tetrac, without a significant change in the BMR (Figure 5). This effect was quantitatively slightly greater than that obtained with trit. As would be expected, the serum PBI level was tremendously elevated out of proportion to the metabolic state, and rose in a roughly linear response to the dose of tetrac employed. In Case 2, 9 mg of tetrac was required to maintain normal BMR's and clinical status, and Case 1 was still slightly hypothyroid on 5 mg per day. When tetrac was discontinued in Case 2, the metabolic response was dissipated rather rapidly; the BMR stabilized at -30 per cent within seven days, the serum cholesterol level rose, and the patient appeared myxedematous two weeks later. The serum PBI level, however, was still 10 μg per 100 ml three weeks after tetrac had been discontinued.

At this time, as illustrated in Figure 6, triprop was started in very small doses, and reproducible BMR's were obtained on successive days before increments were made. On 2 mg per day, the BMR had not appreciably changed, while the se-

rum cholesterol level had decreased 120 mg per 100 ml (Figure 6). The patient was clinically euthyroid on 6 mg of triprop per day. The PBI was again elevated out of proportion to the clinical response, reaching levels approximately equivalent to those obtained with triac, but not as high as those with tetrac, in accord with the amounts of iodine administered.

Case 3 was treated initially with tetprop, starting with doses of 2.0 mg daily, which were gradually increased to a maintenance level of 14 mg daily (Figure 7). Despite prolonged training, this patient continued to have marked variability in his BMR's. There was, however, an obvious response in both the BMR and the serum cholesterol level to 2.0 mg of tetprop daily. The serum cholesterol levels continued to fall, but did not reach normal levels until tetprop was given in doses of 12 mg daily, at which time the patient was clinically euthyroid. There was very little change noted in any parameter when the dose was increased to 14 mg daily. As would be expected, the serum PBI levels were markedly elevated although not as high as those obtained following tetrac administration.

Both Cases 1 and 2 were subsequently treated with thyroxine and desiccated thyroid. On 0.3 mg of thyroxine they were clinically euthyroid and the BMR and serum cholesterol levels remained normal, although the serum PBI levels were again slightly elevated out of proportion to the metabolic state. The daily oral dose of desiccated thyroid

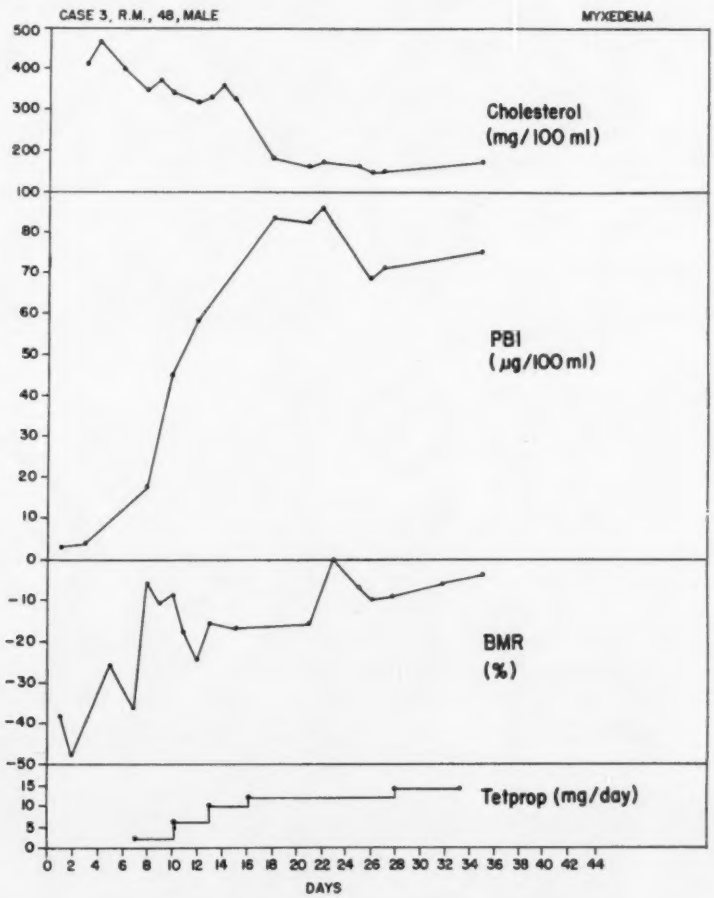

FIG. 7. THE EFFECT OF TETPROP ON THE BASAL METABOLIC RATE, SERUM CHOLESTEROL LEVEL, AND SERUM PROTEIN-BOUND IODINE LEVEL OF A PATIENT WITH PRIMARY HYPOTHYROIDISM.

TABLE II

*Effect of repeated injections of thyroactive substances on metabolism of tissues from thyroidectomized rats**

Substance injected	Thyroxine	Trit	Tetrac	Triac	Tetprop	Triprop
Dose (*mg/kg*)†	1.0	0.25	2.0	0.6	6.0	2.0
No. of animals	6	5	4	4	6	4
Tissue	%	%	%	%	%	%
Kidney	35.9	31.8	22.9	36.9	17.8	36.6
Liver	36.7	40.1	30.8	30.9	31.3	39.8
Heart	38.6	51.6	22.2	28.4	38.6	42.0
Diaphragm	19.8	24.3	17.9	15.1	23.3	18.4
Skel. musc.	31.8	32.2	20.6		15.9	
Pancreas	39.1	37.4	34.0		39.1	
Saliv. gl.	32.9	35.5	27.6	14.2	29.4	22.8
Average	33.5	36.1	25.1	25.1	27.9	31.9
Per cent‡	100	431.1	37.5	127.5§	13.9	52.8§

* Data are expressed as per cent increase above control tissue oxygen consumption.

† Milligram per kilogram of body weight injected subcutaneously once daily for four days; tissue metabolism studied on fifth day.

‡ Relative activity compared to thyroxine as 100 per cent.

§ Based on average of corresponding five tissues.

required to maintain euthyroidism with normal BMR's and serum PBI and cholesterol levels in these two patients ranged from 90 to 120 mg.

DISCUSSION

Gross and Pitt-Rivers (2) found trit to have three to five times the potency of thyroxine in the goiter prevention assay in rats (35). Subsequent work has confirmed a high level of activity (36–38). In this laboratory, we have found that the injection of 0.25 mg trit per kg animal body weight produces the same metabolic stimulation as 1.0 mg. L-thyroxine, as shown in Table II. The metabolic response is due to that of tissues such as liver, kidney, skeletal muscle, heart and the exocrine glands. Other tissues such as brain, spleen, gastric smooth muscle, gonads and accessory reproductive structures of both sexes show no change in oxygen consumption. The pattern of metabolic response is thus the same for triiodothyronine as it is for thyroxine. In man (32, 39–41), trit has been shown to produce all of the metabolic effects of thyroxine. As one would anticipate from the low dose levels required, the serum PBI levels remain in the hypothyroid range when the patient is clinically euthyroid (33, 42). Starr, Snipes and Liebhold-Schueck (34) have demonstrated that trit is adequately measured by the Barker PBI method, and conclude that it is rapidly utilized by the tissues and leaves the blood promptly. In the present study, both patients were slightly hypermetabolic on a daily oral dose of 100 μg of trit, and were euthyroid on 90 μg daily. It is apparent that serum cholesterol levels decreased on small doses of trit before there was a significant change in BMR (Figure 3).

Pitt-Rivers and Harrington synthesized and studied both triac and tetrac in the goiter-prevention assay and found that both had a potency of about 0.1 that of triiodothyronine (4, 43). Other studies (Table III) have shown variation in the relative potencies of these analogs. Thibault and Pitt-Rivers (5) reported that triac and tetrac had a profound effect on biological oxidations both *in vitro* and *in vivo*. The results reported in the present study do not confirm these *in vitro* effects nor the immediate effects *in vivo*. However, it is clear that these compounds do have biological properties similar to thyroxine. Although the acetic acid analogs have been demonstrated to be normal products of thyroxine and trit degradation (10–17), there is no clear basis

TABLE III

Relative potencies of thyroactive substances in rats, obtained by various assay technics, and in man

	Summated tissue response in rats	Human	Maintenance	Goiter prevention in rats*	Calorigenic action in rats*	Thyroid I^{131} uptake block in rats†
	%	%	*mg*	%	%	%
L-Thyroxine	100	100	0.3	100	100	100
L-Trit	431	333	0.09	800	800	300
Triac	128	5	6.0	51	21	75
Tetrac	38	3	9.0	57	11	75
Triprop	53	5	6.0	28	10	60
Tetprop	14	2.5	12.0	14	7	60

* Data from Stasilli, Kroc and Meltzer (37).

† Data from Money, Meltzer, Feldman and Rawson (38).

for assuming that this change is obligatory before the amino acid forms of the hormones can be active.

Lerman and Pitt-Rivers first used triac in the treatment of myxedema (44), giving it daily by intravenous injection in doses of 0.1 to 1.0 mg. Clinical improvement was noted, along with loss of weight and reduction in the serum cholesterol level. There was little alteration in basal metabolic rate. However, when the dose was increased to 4 mg daily the basal metabolic rate returned to normal (19). Trotter, employing 6 mg of triac daily, produced clinical recovery and a return of the basal metabolism to normal levels in a case of myxedema (45). Similar findings have been reported by other investigators, (9, 26, 28, 29). Both patients in the present series receiving triac were clinically euthyroid on 6 mg daily. When small doses were given, a depression of the serum cholesterol level was again demonstrated without a concomitant increase in BMR. The serum PBI level was elevated out of proportion to the clinical state, as one might expect from the large amounts of the drug which are required to maintain euthyroidism.

Goolden found that it required 8 mg orally per day of tetrac to maintain clinical euthyroidism, and that a therapeutic response was evident within 48 hours (27). He also noted that the serum cholesterol level fell on doses less than that needed for maintenance of euthyroidism.

Other workers have confirmed the metabolic activity of this compound (9, 26–29). In the present study tetrac was also found to be a relatively weak thyroactive substance in man, since 9 mg per day was required to restore euthyroidism. At this dose level, the serum PBI was 141 μg per 100 ml, and rose in a roughly linear response to the dosage employed (Figure 5). On small doses the serum cholesterol level fell without elevation of the BMR. On discontinuance of therapy, one patient was markedly myxedematous at the end of the second week, although the serum PBI level was still slightly elevated.

It is apparent from the present studies in man that, although they are less active, both triprop and tetprop also have metabolic properties similar to thyroxine. The effects of triprop and triac were comparable, the approximate daily dose for maintenance of euthyroidism being 6 mg in both instances. Tetrop was considerably less active than triprop and slightly less active than tetrac, since 12 mg daily was required to maintain euthyroidism (Table III). Maintenance doses of thyroxine and desiccated thyroid in these patients were established at levels of 0.3 mg and 120 mg daily, respectively.

These studies illustrate that a dissociation of cholesterol and metabolic rate responses may be obtained with trit, thyroxine, triac, tetrac, triprop and tetprop when small enough doses are used. Although this may be a phenomenon shared by all thyroactive substances, it is conceivable that an even more marked dissociation of responses might be obtained by suitable alterations of the thyroxine molecule. Such an achievement has already been approached in the specific case of tadpole metamorphosis, where tetprop and triprop exhibit 120 and 290 times, respectively, the activity of thyroxine (46). This possibility should also be viewed along with the now well established dissociation of responses obtained by altering the molecular structure of the adrenal cortical steroids (47–49).

No peculiarly specific role of the acetic acid analogs in thyroid physiology has been established. It seems unlikely that these weakly thyroactive compounds are obligatory forms of the thyroid hormone utilized by the tissues. Since amino acids in general are broken down through oxidative deamination and decarboxylation, the thyroacetic compounds would be expected as metabolic degradation products of thyroxine and trit. The same cannot be said of the propionic acid analogs, and they should probably be considered as the outstanding contemporary example of purely synthetic thyroactive substances.

SUMMARY

Studies have been done to evaluate, both in animals and in man, the metabolic actions of the acetic and propionic acid derivatives of L-thyroxine and 3,5,3′-triiodo-L-thyronine. The effects of these substances were compared, both qualitatively and quantitatively, with those of the parent compounds. The acetic acid analogs produced an acceleration of oxygen consumption in animal tissue preparations only when they were preinjected in the animals. Both the acetic and propionic acid analogs

were weakly thyroactive in man, with 5 per cent or less of the activity of thyroxine. All of the analogs had metabolic properties similar to the parent compounds. A dissociation of cholesterol and metabolic rate responses was obtained with all compounds, when small enough doses were used.

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CLINICAL, MORPHOLOGICAL AND BIOCHEMICAL STUDIES OF A VIRILIZING TUMOR IN THE TESTIS *

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Although there is considerable indirect evidence indicating that virilizing testicular tumors exert their effects by producing androgenic steroid hormones, few attempts have been made to determine the exact nature of the compounds produced. One type of tumor which exerts particularly marked virilizing effects when found in prepubertal boys is that classified as an interstitial cell tumor of the testes (1–21). It has been well established that such tumors cause elevated levels of urinary 17-ketosteroids. The morphology of these tumors leads one to predict that they are producing testosterone, since normal interstitial cells seem to have this function (22, 23). A few studies of the qualitative nature of the urinary steroids in patients with this type of tumor seem to confirm such a conclusion (13, 24). Accordingly, when the opportunity arose to study a patient bearing a testicular tumor thought to be of this type, it seemed worthwhile to attempt to establish the exact nature of the steroids produced. The study presented here was therefore undertaken.

This paper presents a description of the patient, histological observations on the tumor, the results of urinary steroid assays and the results of experiments in which slices of the tumor were incubated with C^{14}-labeled steroid precursors.

EXPERIMENTAL AND RESULTS

I. Clinical data

D. W. (Duke no. D-99663), a 5-year-and-7-month old white male, was first seen at Duke Hospital on November 1, 1954, because of precocious growth and development, increasing libido, and personality changes. His birth and early development had been normal, but at 3 years of age progressive statural growth spurt and enlargement of the penis were noted. At 4 years of age, genital hair appeared, followed shortly by axillary, body and facial hair, mild facial acne and progressive deepening of the voice. During one year a gradual, painless enlargement of the right testis had been noted, accompanied by increasingly frequent penile erections and masturbation, apparently without ejaculation. The child became aggressive, somewhat withdrawn and uninterested in previous playmates and activities.

Physical examination revealed normal vital signs with a blood pressure of 112/60 mm Hg. His height was 135

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FIG. 1. THE PATIENT PRIOR TO OPERATION; AGE, FIVE YEARS AND SEVEN MONTHS.

cm and his weight 33.6 kg. Anthropometric measurements corresponded to those of an average boy approximately 11 years of age. The muscular development was rather Herculean (Figure 1). The dental age was estimated to be 6.5 years. There was slight facial acne, moderate axillary hair, and abundant heavily pigmented and coarse pubic hair. Hirsutism was noted over the extremities, back and upper lip. The penis, in a flaccid state, measured 11.5 cm in circumference and 12.5 cm in length. The right testis was enlarged ($4.5 \times 3.7 \times 3.2$ cm). It was uniformly abnormally firm, slightly irregular in contour and freely movable within the well rugated and pigmented scrotum. The cord structures on that side were normal to palpation, and there was no local or distant lymphadenopathy. The left testis was normal in size, shape and consistency ($2 \times 0.7 \times 0.7$ cm). The prostate was enlarged and comparable in size to that of an adolescent. No other physical abnormalities were noted.

TABLE I

Urinary steroid and gonadotropin excretion

	Preoperative	Postoperative 2 days	Postoperative 1.5 years
	mg/24 hrs	*mg/24 hrs*	*mg/24 hrs*
17-Ketosteroids (25)*	13.6	1.4	1.6
Neutral acylable lipids (26)*	138	50	
Fluorogenic phenols (27)*	78	80	13
Pregnanetriol†	4.1	0.4	
Corticosteroids (28)‡	1.4	1.1	1.1
Gonadotropins§	<1		7

* Estimations carried out on extracts of urine boiled with 15 volumes per cent hydrochloric acid for ten minutes.
† Carried out in the laboratory of Dr. Judson J. Van Wyk.
‡ Estimations carried out on extracts of urine incubated with mammalian β-glucuronidase (Ketodase, Warner-Chilcott Laboratories) at pH 5.1.
§ Rat uterine units per 24 hours.

TABLE II

Urinary levels of individual 17-ketosteroids

	Preoperative	Postoperative 2 days
	mg/24 hrs	*mg/24 hrs*
Androsterone	2.4	0
Etiocholanolone	2.0	0.1
Androstane-3,17-dione and etiocholane-3,17-dione	0.1	0.1
Dehydroepiandrosterone	1.4	0
11β-Hydroxyetiocholanolone [and $\Delta^{9(11)}$-etiocholenolone]	0.2	0
11-Ketoetiocholanolone	0.2	0
11β-Hydroxyandrosterone [and $\Delta^{9(11)}$-androstenolone]	1.6	0
11-Ketoandrosterone	0.2	0.1

On psychological consultation the child was thought to be advanced both intellectually and in his social adjustments, corresponding approximately to the age of 8 years. The intelligence quotient was 135 and the social quotient 146.

The hemogram and urinalysis were normal, and the serological test for syphilis was negative. Blood chemical analyses (nonprotein nitrogen, total proteins and albumin/globulin ratio, fasting blood sugar, serum cholesterol and electrolytes) were normal. The basal metabolic rate was + 3 per cent. X-rays of the chest and skull and intravenous pyelograms revealed no abnormali-

FIG. 2. THE SECTIONED SURFACE OF THE TUMOR, TESTIS AND CORD STRUCTURES ARE ILLUSTRATED. The identification tag is 2 cm in length.

FIG. 3. THE MARGIN OF THE TUMOR IS SHOWN AT ITS JUNCTION WITH THE SURROUNDING NORMAL TESTICULAR TUBULES. Note the fibrous septa dividing the tumor into lobules. (Hematoxylin and eosin, ×205.)

ties. The bone age was greater than 10 but less than 14 years. Urinary steroid and gonadotropin analyses are reported below (Tables I and II).

On November 16, 1954, a right radical orchiectomy and biopsy of the left testis were performed.

Pathological anatomy. Gross: The right testis measured 2.5 × 3.0 × 3.5 cm and weighed 25 g. On section a grossly well encapsulated, homogeneous soft tumor, lobulated by delicate fibrous septa, was observed (Figure 2). The sectioned surface of the tumor measured 23 × 32 mm, bulged and was dark brown with a distinct green cast. The surrounding testicular tissue was compressed.

Microscopic examination. There was an incomplete, thin connective tissue capsule. Where the capsule was incomplete, scattered small clumps of tumor cells extended for a short distance into the surrounding tissue. The tumor was made up of solid sheets of cells broken up by occasional thin, vascular, fibrous tissue septa (Figure 3). A delicate reticulum supporting framework further separated the tumor into clumps of 2 to 12 cells. The tumor cells were irregular, polyhedral in shape, 14 to 35 μ in diameter (average, 22 μ), and occasionally had indistinct cell boundaries, although in most areas the cell boundaries were very clearly defined. Occasionally, syncytial masses of cytoplasm were formed with 2 to 5 nuclei. The nuclei were round, vesicular and showed no wrinkling of the nuclear membrane. There was a fine granular chromatin network with 1 to 3 prominent nucleoli. Occasional large hyperchromatic nuclei were present, but generally there was little variation and mitoses were rare. With Helly's fixation the cytoplasm was brightly eosinophilic, whereas with 10 per cent formalin fixation the cytoplasm appeared light pink with prominent unstained vacuoles.

The most striking cytological feature was the presence of a variety of cytoplasmic inclusions. With hematoxylin and eosin and Helly's fixation most of the cells showed very finely divided, granular, brown inclusions clustered about the nucleus (Figure 4). This material was indistinguishable from the "lipochrome" pigment present in normal interstitial cells; however, it was not sudanophilic, nor was there any sudanophilic material in any of the cells. This material reacted positively with the Schiff reagent and was particularly well demonstrated by phosphotungstic acid hematoxylin stains. A second type of cytoplasmic inclusion was present in most of the cells. These structures varied considerably in size measuring from 1.7 to 5.1 μ in diameter. They were usually clus-

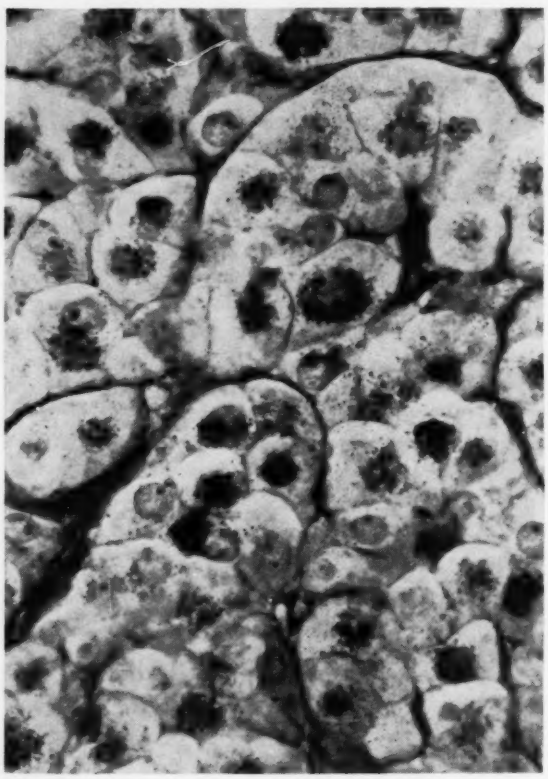

FIG. 4. THIS MICROPHOTOGRAPH ILLUSTRATES THE LIPOCHROME GRANULES CLUSTERED ABOUT THE NUCLEUS OF VIRTUALLY EVERY CELL. (Periodic acid-Schiff, ×1,021.)

tered about the periphery of the cytoplasm and occasionally filled the entire cell. They were highly refractile and were surrounded by a clear halo. Some of these granules had an internal structure in which the outer wall was stained dark blue with phosphotungstic acid hematoxylin. Just internal to this was a narrow, clear, unstained area. A solid mass of dark blue material made up the remainder of the inclusion. Others were solid and homogeneous. These were not well seen in formalin-fixed tissue by any technique used but were readily apparent in Helly-fixed tissue, particularly with Masson trichrome and phosphotungstic acid hematoxylin stains as well as with hematoxylin and eosin (Figure 5). A third type of inclusion was present, consisting of very rare rod-shaped structures which were indistinguishable from crystalloids of Reinke. Their most striking tinctorial reaction was their oxyphilia rendering them red with the Masson trichrome technique. Some of these were seen crossing a cell boundary and extending into an adjacent cell; others lay completely within the cytoplasm of a single cell (Figure 6).

The seminiferous tubules and interstitial cells of the left testis showed development corresponding to the patient's chronological age. There was no evidence of spermatogenesis. The pathological diagnosis was interstitial cell tumor of the right testis.

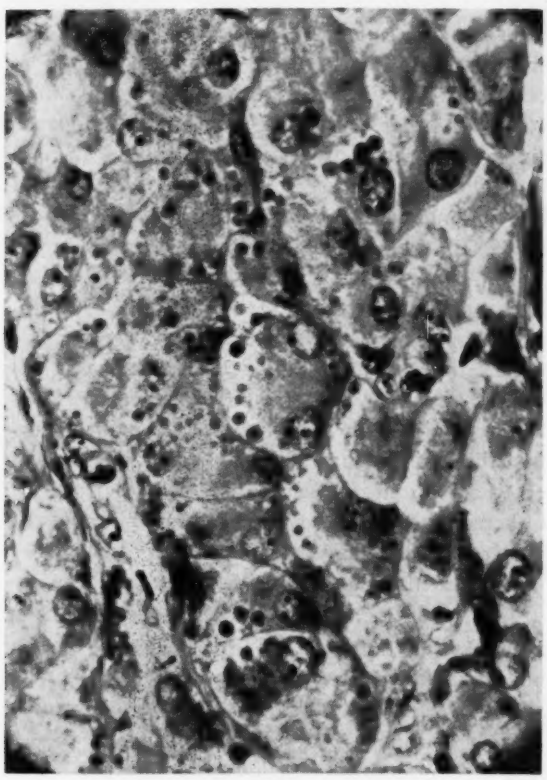

FIG. 5. LARGE CYTOPLASMIC GRANULES CAN BE SEEN DISTRIBUTED ABOUT THE PERIPHERY OF THE TUMOR CELLS. Nuclear detail is also well shown. (Phosphotungstic acid hematoxylin, ×1,021.)

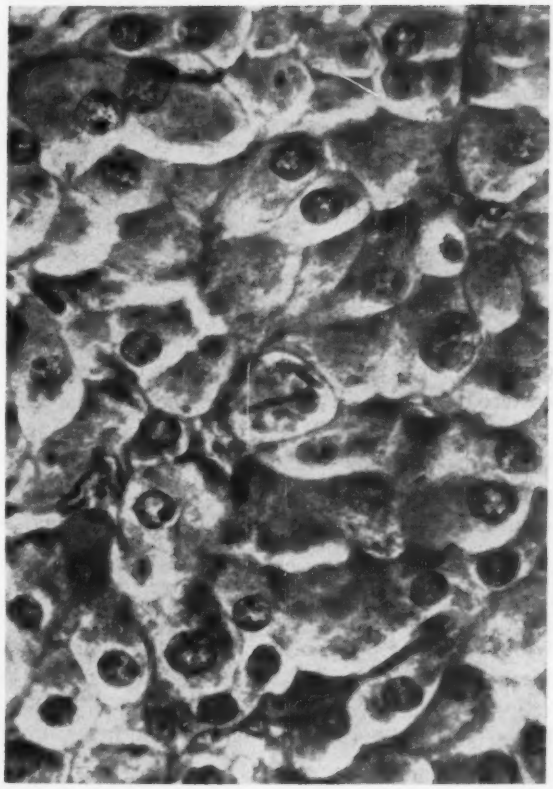

FIG. 6. THE CELL IN THE CENTER OF THE FIELD CONTAINS TWO ELONGATED CRYSTALLOIDS OF REINKE. (Masson trichrome, ×1,021.)

Follow-up. Since discharge from the hospital there has been no regression in any of the somatic features previously depicted. In March and June, 1955, no essential differences were noted. While the left testis was normal in size and consistency, the strong sexual drive persisted. The latter was considerably reduced by May, 1956, and the child's social adjustment was better. His height was 149 cm and his weight 39.5 kg, gains of 14 cm and 5.9 kg, respectively, since operation. At this time it was noted that the remaining left testis had grown remarkably in size in the preceding year, now measuring 5.5 × 3 cm. The testicular size, consistency and contour, and the prostate gland, were now comparable to that of a normal late-adolescent male. He was last seen in November, 1956, two years after surgery, with findings similar to those noted six months earlier except for a further gain of 6 cm in height. The bone age was estimated as 12 years, an advance of almost two years since operation. Daily urinary gonadotropin excretion was 7 rat uterine units, within the range for a normal adult. The postoperative urinary steroid and gonadotropin levels are recorded in Tables I and II.

II. Urinary steroids

Estimations of steroid concentrations were carried out on 24-hour urine specimens collected before and at various periods after the removal of the tumor. The values obtained are shown in Table I. These values as well as those of gonadotropins are in agreement with values previously observed in this syndrome. The excretion of corticosteroids measured by the Silber and Porter method (28) was in the normal range for a 5 year old boy and was unchanged after removal of the tumor. Paper chromatograms of the corticosteroid fraction in the toluene/methanol: water (75:25) system (29) showed the presence of tetrahydrocortisone and tetrahydrocortisol as the preponderant corticosteroids before and after the operation.[1] No unusual reducing compounds were detected.

The daily excretion of 17-ketosteroids, neutral acylable lipids (hydroxysteroids) and pregnanetriol was elevated before removal of the tumor. A further sample of urine was boiled with 15 volumes per cent of hydrochloric acid, and the ether extracts were chromatographed in the Bush ligroin/methanol: water (96:4), and ligroin: toluene (67:33)/methanol-water (70:30) systems. The chromatograms were sprayed with Zimmermann reagent and the amount of each compound estimated by comparison of size and intensity of the spot with the sizes and intensities resulting from known amounts of the compound chromatographed at the same time. This method has been found to be accurate within 15 per cent (30). The daily excretion of individual 17-ketosteroids estimated by this method is presented in Table II, in which the artifacts produced by the hydrolysis with acid are included with their known precursors.

Elevated excretion of both $C_{19}O_2$ and $C_{19}O_3$ compounds, androsterone and 11β-hydroxyandrosterone in particular, is to be noted. The excretion of all steroids measured after removal of the tumor was normal. The level of fluorogenic phenols gave no indication of elevated estrogen excretion and was unchanged by removal of the tumor; the reduction of this level 1.5 years after the tumor was removed is unexplained.

III. In vitro studies

The incubation of slices of this tumor was undertaken as a means of direct biochemical assessment of its steroid production. This technique utilizes small amounts of tissue and C^{14}-labeled steroid precursors such as acetate-1-C^{14}, and is one which is of proven value in this type of study (31). In order to facilitate extraction of the trace amounts of radioactive steroids from the incubation medium and tissue, it is necessary to add known amounts of crystalline nonradioactive steroids. In the case of each substance, the total steroid (radioactive and carrier) may then be isolated and purified and its radioactivity per milligram (specific activity) determined after suitable purification. By calculation back to the original weight of carrier, the amount of radioactivity associated with each carrier steroid may be ascertained. This in turn reflects the *relative* amount of each steroid formed during the experiment. Other methods for the microdetermination of the absolute amounts of endogenous steroid hormones present in endocrine glands have recently been developed (32–36); these however require exacting manipulations for certainty in the identification and quantitative measurement of the minute amounts of the compounds found. The incubation of specific C^{14}-labeled steroids with tissues and the isolation of radioactive products, also by means of carrier dilution, provide an assessment of the tissue's capacity to effect certain specific transformations.

[1] The chemical names for the steroid compounds used in this paper are as follows: 3α-hydroxyandrostan-17-one (androsterone); 3α,11β-dihydroxyandrostan-17-one (11β-hydroxyandrosterone); 11β-hydroxy-4-androstene-3,17-dione (11β-hydroxyandrostenedione); 4-androstene-3,11,-17-trione (adrenosterone); 4-androstene-3,17-dione (androstenedione); 3α-hydroxyetiocholane-17-one (etiocholanolone); 17β-hydroxy-4-androsten-3-one (testosterone); 11β,17β-dihydroxy-4-androsten-3-one (11β-hydroxytestosterone); 17β-hydroxy-4-androstene-3,11-dione (11-ketotestosterone); 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone); 3α,17α,21-trihydroxypregnane-11,20-dione (tetrahydrocortisone); 3α,11β,17α,21-tetrahydroxypregnan-20-one (tetrahydrocortisol); pregnane-3α,17α,-20α-triol (pregnanetriol); 4-pregnene-3,20-dione (progesterone); 17α-hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone); 11β-hydroxy-4-pregnene-3,20-dione (11β-hydroxyprogesterone); 6β-hydroxy-4-pregnene-3,20-dione (6β-hydroxyprogesterone); 6β-acetoxy-4-pregnene-3,20-dione (6β-acetoxyprogesterone); 3β-hydroxy-5-pregnen-20-one (5-pregnenolone).

The chemical methods employed consisted first in making a total lipid extract which was separated into nonpolar (fatty constituents, and so forth) and polar lipids. The latter fraction containing the carrier steroids was separated into acidic, phenolic and neutral polar lipids, and on occasion, into ketonic and nonketonic fractions. These procedures, described below in greater detail, afforded extracts containing the steroids relatively free from extraneous material. These extracts were then subjected to countercurrent distributions (ccd) and paper chromatographic separations in a variety of systems. Each steroid so isolated was free from steroidal and other contaminants, and was judged *chemically pure* by melting point and other criteria. The achievement of radiochemical purity required further procedures, which are described below.

METHODS

I. General

Countercurrent distributions. All 4- and 8-transfer distributions were carried out in separatory funnels. Lower layers were transferred, and equal volumes of the two phases were used. The 24-transfer distributions were carried out in a 25-tube, stainless steel Craig apparatus and those of greater numbers of transfers were carried out in the glass apparatus (H. O. Post Scientific Instrument Co.).

Paper chromatography. Chromatograms of the Zaffaroni type (37) and modifications (38, 39) were run at room temperature on Whatman no. 1 filter paper. In all systems employed, propylene glycol was used as the stationary phase. The following solvents were used as moving phases: ligroin (38), toluene (37), toluene: cyclohexane (50: 50), and toluene: methyl cyclohexane (50: 50) (39). Chromatograms of the type described by Bush (29) were run at 37° C on Whatman no. 2 filter paper.

Compounds were located on the developed chromatograms by visualization with ultraviolet light (Δ^4-3-ketosteroids), and by spraying with alkaline blue tetrazolium (reducing steroids) (40), alkaline *m*-dinitrobenzene (all ketosteroids) (38), or antimony trichloride reagent (Δ^5-3β-hydroxysteroids) (41). After locating the steroids on the preparative chromatograms, the zones containing compounds or mixtures of compounds were cut out, cut into small pieces, and extracted repeatedly with hot methanol, then chloroform, or a mixture of equal parts of methanol and chloroform. The combined extracts were filtered to remove paper fibers and evaporated to dryness under reduced pressure. The extracts of paper previously saturated with propylene glycol were dissolved in chloroform and the chloroform washed several times with 1/10 volumes of water to remove the glycol.

When required, zones of radioactivity on the chromatograms were located by means of contact radioautography. This consisted in placing the chromatogram (or a representative portion) in contact with Kodak Medical X-ray film (no screen) for the required exposure time (42), and developing the film in the usual manner.

Measurement of radioactivity. All samples were plated at infinite thinness in metal dishes and counted in a windowless gas-flow counter. A minimum of 2,000 counts was accumulated on each. All counts were corrected for background.

Ultraviolet absorption. All ultraviolet absorption spectra and values of absorbance were measured in 95 per cent ethanol.

Formation of derivatives. Acetylations were carried out at room temperature with acetic anhydride and anhydrous pyridine (1: 1); excess reagents were evaporated under a stream of air. Oxidations were carried out with chromium trioxide in glacial acetic acid at room temperature for two hours (43). Formation of oximes was carried out in the conventional way.

II. Incubation

The tumor was received within a few minutes of excision and chilled in cold physiological saline. It was sectioned and a representative portion used for the biochemical studies; the remainder was taken for pathology. Slices were prepared with a Stadie-Riggs slicer and distributed among four incubation flasks. These flasks contained as their final contents 0.93 g of tumor tissue, 20 ml of fresh human serum, 0.02 mmole of potassium fumarate, 10 mg of glucose, 500 units of pregnant mare serum gonadotropin (Equinex-Ayerst), 500 units of pregnancy urine gonadotropin (APL-Ayerst) and 5 units of porcine pituitary gonadotropin (FSH-Armour). The radioactive substances (the first three shown to be chromatographically homogeneous), added one to each flask, were: A) testosterone-4-C^{14} (0.92 mg, 3.82×10^6 cpm); B) progesterone-4-C^{14} (2 mg, 350,000 cpm); C) cholesterol-4-C^{14} (2.2 mg, 7.5×10^6 cpm); and D) sodium acetate-1-C^{14} (1 mc); the radioacetate was added dissolved in water, and the steroids in 0.2 ml of propylene glycol. Incubation was carried out with shaking for three hours at 37° C with 95 per cent O_2 and 5 per cent CO_2 as gas phase. The flask contents were frozen until analyzed.

III. Addition of carrier steroids, extraction and preliminary purification

On removal of the incubation mixtures for storage, chromatographically pure steroids in varying amounts (5 to 20 mg) were added to each of the incubation flasks (Table III).

Each incubation mixture (tissue, medium and added carrier) was extracted individually in a Waring blendor with 25 volumes of chloroform: methanol (2: 1) mixture (44). After removal of tissue debris by filtration, 1/5 volume of water was added and the mixture shaken; the upper aqueous layer was removed and extracted twice with 1/2 volume of chloroform. The combined lower

TABLE III

Carrier steroids added to incubation mixtures

Steroids	Experiment—C^{14}-substrate				
			C. Cholesterol*		
	A. Testosterone	B. Progesterone	I	II	D. Acetate
	mg	*mg*	*mg*	*mg*	*mg*
Estrone					5
Estradiol-17β					5
Estriol					5
Testosterone	10	10	20		10
4-Androstenedione	10	10	20		10
11β-Hydroxyandrostenedione	10	10†	20		10
11β-Hydroxytestosterone	10		20		
Adrenosterone	10				5†
Dehydroepiandrosterone				20	10
5-Androstene-3β,17β-diol				20	
Progesterone		10	20		10
17α-Hydroxyprogesterone		10	20		5†
11β-Hydroxyprogesterone		10†			5†
11-Ketoprogesterone		10†			
5-Pregnenolone				20	10
3β,17α-Dihydroxy-5-pregnen-20-one				20	
6β-Hydroxyprogesterone					5†

* The extract from this incubation was divided into two equal parts; estrogens were added before the division and the two resulting phenolic fractions were combined later in the fractionation; other carriers were added after division of the extract.

† Added at a later stage in the fractionation procedure; for details see text.

layers were evaporated to dryness and the residue subjected to an 8-transfer ccd between ligroin (bp 65 to 75° C) and 90 per cent aqueous methanol. The individual tubes from the distribution of each incubation were evaporated to dryness, weighed, assayed for radioactivity and for the carrier ketosteroids (by absorption at 240 mμ or by the antimony trichloride reagent). The tubes containing the carrier steroids and associated radioactive polar material from each incubation were combined and dissolved in toluene. Each pooled sample was separated into the respective phenolic, acidic and neutral fractions by shaking the toluene solution with 1 N sodium hydroxide (27). The aqueous alkali solution was adjusted to pH 8.5 and shaken with ether to remove the phenols. The neutral material remained in the toluene solution. The neutral polar fractions obtained in the incubations of testosterone (A), progesterone (B) and cholesterol (C) were subjected to a separation with Girard reagent T into ketonic and nonketonic fractions. The extract from the acetate-1-C^{14} experiment (D) was not subjected to this separation.

IV. Separation and purification of carrier steroids

Since somewhat different methods were used for the purification of the individual carrier steroids in each experiment, they will be discussed separately. The steps involved in establishing the radiochemical purity (45) of each apparently labeled compound will be presented for each compound. Where derivatives of the carriers were prepared (acetates, oximes, and so forth) the values of specific activity, unless otherwise noted, are not corrected for changes in molecular weight, and are given as determined.

V. Incubation experiments

EXPERIMENT A. Incubation of testosterone-4-C^{14}: The ketonic fraction from this incubation was chromatographed in ligroin/propylene glycol for 96 hours. Each steroid carrier zone was located and recognized by its relative position (37), eluted and rechromatrographed. 17α-Hydroxyprogesterone, testosterone and adrenosterone were not completely separated by this chromatography. Separation was carried out by acetylation of the mixture and the complete removal of testosterone acetate by chromatography in ligroin/propylene glycol; the two remaining steroids were separated by chromatography on paper in toluene: methyl cyclohexane (50: 50)/propylene glycol.

11β-Hydroxytestosterone: This compound did not migrate from the starting line as the free steroid in ligroin/propylene glycol in 100 hours. It was chromatographed in toluene/propylene glycol for 12 hours to yield a contaminated product, 3.7 mg in weight and specific activity 7,370 cpm per mg. One and two-tenths mg of this was diluted with 30 mg of 11β-hydroxytestosterone carrier, acetylated, and chromatographed in ligroin/propylene glycol (24 hours), to provide a diluted 11β-hydroxytestosterone acetate (mp 151 to 152° C, from acetone: hexane) with a specific activity of 114 cpm per mg (or 129 corrected to the free compound). Oxidation with chromic acid and chromatography in ligroin/propylene glycol gave 11-ketotestosterone acetate (mp 164 to 165° C, acetone: hexane) with a specific activity of 112 cpm per mg (127 as the free compound). Saponification with potassium carbonate in ethanol (46) gave 11-ketotestosterone (mp 184 to 185° C); specific activity 131 cpm per

mg (130 as 11β-hydroxytestosterone). The specific activity of the initially isolated 11β-hydroxytestosterone was calculated as 3,300 cpm per mg; this value is used in the calculation of those data for this compound in Table IV.

11β-Hydroxyandrostenedione: As initially isolated and crystallized (mp 194° C), this compound had a specific activity of 6,500 cpm per mg. Two samples of 0.5 mg each were diluted with 50 and 70 mg of carrier and purified as follows. Sample 1 was chromatographed (ligroin/propylene glycol, 116 hours) and recrystallized from acetone: hexane (59 cpm per mg); oxidized to adrenosterone, chromatographed, and recrystallized from methanol (mp 220 to 225° C; 60 cpm per mg); recrystallized from acetone: hexane (mp 219 to 220° C; 59 cpm per mg); converted to the dioxine and recrystallized from methanol (mp 209 to 210° C; 56 cpm per mg, corrected to the molecular weight of adrenosterone). Sample 2 was chromatographed and crystallized (48 cpm per mg); oxidation followed by chromatography and crystallization gave adrenosterone (mp 218° C; 46 cpm per mg). The initially isolated steroid was radiochemically pure at 6,500 cpm per mg.

Adrenosterone: As isolated and recrystallized (mp 212° C) the specific activity was 278 cpm per mg and as may be seen from the following, was slightly contaminated. The dioxime (mp 222° C; 258 cpm per mg) was chromatographed (toluene/propylene glycol, 34 hours) and recrystallized from methanol (mp 218 to 220° C; 190 cpm per mg, calculated as adrenosterone, 209 cpm per mg); the material in the mother liquor had the same specific activity. Adrenosterone was considered pure at 209 cpm per mg.

Androstenedione: This compound was recrystallized from ethyl acetate to give crystals (mp 172 to 173° C; 19,700 cpm per mg) and a crystalline residue from the mother liquors (17,400 cpm per mg). This product was not further manipulated and was considered radiochemically pure.

Testosterone: The original substrate, diluted as noted, was recovered (mp 151 to 152.5° C; 250,000 cpm per mg).

Radioautography of the initial chromatograms having revealed no zones other than those associated with the above steroids, it is presumed that no other important transformations of testosterone occurred in this study. Total radioactivity associated with each carrier steroid at the above specific activities is given in Table IV, column A, corrected to the amount present at the addition of the original carrier.

EXPERIMENT B. Incubation of progesterone-4-C^{14}: The chromatography of the neutral ketonic fraction from this incubation was somewhat encumbered by the presence of a strongly fluorescing material whose presence seriously distorted the initial chromatograms in ligroin/propylene glycol. In order to eliminate this material repeated chromatography was necessary; quantitative measurement of several carrier steroids showed that but 1.0 to 1.2 mg remained. This was assumed to be true for all others and at this point additional amounts of each carrier were added (10 mg) to the recombined fractions, and all were rechromatographed in ligroin/propylene glycol, and separated as described above; 11β-hy-

TABLE IV

*Results of incubation studies**

Added carriers	Experiment—C^{14}-substrate					
	A. Testosterone		B. Progesterone		D. Acetate	
	3.82×10^6 cpm		3.5×10^5 cpm		1.3×10^9 cpm	
	cpm	%	*cpm*†	%	*cpm*	%
Estrone					None	
Estradiol-17β					None	
Estriol					None	
Testosterone	2.500×10^6	66.0	0.11×10^5	3.1	0.30×10^5	0.003
4-Androstenedione	0.197×10^6	5.0	0.30×10^5	8.6	6.50×10^5	0.065
11β-Hydroxyandrostenedione	0.065×10^6	1.7	0.13×10^5	3.7‡	3.47×10^5	0.035
11β-Hydroxytestosterone	0.033×10^6	0.8				
Adrenosterone	0.002×10^6	0.05			0.06×10^6	0.0006‡
Dehydroepiandrosterone					0.04×10^6	0.0004
5-Androstene-3β,17β-diol						
Progesterone			2.03×10^5	58.0	None	
17α-Hydroxyprogesterone			1.14×10^5	32.6	0.63×10^5	0.006‡
11β-Hydroxyprogesterone			None		None	
11-Ketoprogesterone			None			
5-Pregnenolone					None	
3β,17α-Dihydroxy-5-pregnen-20-one						
6β-Hydroxyprogesterone					None	
Total		74		106		0.11

* Amount of radioactivity and per cent of added substrate isolated by carrier-dilution.

† These values are based upon a further dilution of the original carriers by a ratio of 1 to 10; this dilution is explained in the text.

‡ This value is not comparable to others, as carrier was added late in the fractionation scheme.

droxyandrostenedione, 11β-hydroxy- and 11-ketoprogesterone were added at this point for the first time.

11β-Hydroxyandrostenedione: This compound was recognized as a transformation product of progesterone-C^{14} late in the study and was seen first as a radioactive zone of slow mobility [slower than 11β-hydroxyprogesterone and 17α-hydroxyprogesterone (38)]. To the material eluted from this radioactive area, 5 mg of carrier 11β-hydroxyandrostenedione was added and the whole rechromatographed in ligroin/propylene glycol; the resulting zone was eluted and the steroid recrystallized from acetone: hexane to constant specific activity (mp 195.5 to 196.5° C; 127 cpm per mg); 2.57 mg of this material was diluted with 4.38 mg of additional carrier and oxidized, chromatographed, and crystallized from methanol to give adrenosterone (mp 213 to 214° C; 47 cpm per mg). The dioxime was formed and had a specific activity of 43 cpm per mg. The 11β-hydroxyandrostenedione obtained after the initial addition of carrier was considered radiochemically pure at approximately 127 cpm per mg. Comparison of specific activity with those of other isolated compounds is not valid because the carrier in this case was not added at the same stage as in the others. The total amount of this compound formed, shown as counts per minute in Table IV, must be considered minimal.

11β-Hydroxyprogesterone: As isolated following the second addition of carriers, this compound (14 mg) had a specific activity of 44 cpm per mg but was contaminated with traces of 17α-hydroxyprogesterone. Recrystallization from ethyl acetate gave 10.4 mg (mp 182.5 to 183.5° C; 8 cpm per mg). Oxidation with chromic acid followed by chromatography and crystallization from methanol gave 11-ketoprogesterone (mp 170.5 to 172.5° C; 5 cpm per mg). Formation of the oxime and recrystallization (ethyl acetate) gave crystals (mp 226 to 229° C) counting 2 cpm per mg. 11β-Hydroxyprogesterone was therefore considered to be devoid of significant radioactivity.

17α-Hydroxyprogesterone: Nine and five-tenths mg of crystalline steroid was isolated counting 1,050 cpm per mg. Of this, 1.85 mg was diluted with 18 mg of additional carrier and the oxime formed and recrystallized from methanol (mp 244 to 246° C). The oxime had a specific activity of 96 cpm per mg; the mother liquors yielded a residue which had essentially the same specific activity. 17α-Hydroxyprogesterone was considered pure at 1,050 cpm per mg specific activity.

Testosterone: Separated by chromatography from the above as the acetate (mp 139.5 to 140.5° C), this compound counted 109 cpm per mg, and was slightly contaminated. Saponified to testosterone (mp 149 to 150° C), the compound counted 104 cpm per mg. Converted to testosterone oxime (mp 212 to 214.5° C), the compound counted 95 cpm per mg. Testosterone was therefore considered radiochemically pure at 104 cpm per mg.

11-Ketoprogesterone: This compound as isolated and crystallized (methanol, mp 171 to 172° C) had a specific activity of 6 cpm per mg. Formation of the oxime and crystallization eliminated the radioactivity.

Androstenedione: This compound was freed of *most* of its contaminating progesterone-4-C^{14} by the second chromatography in ligroin/propylene glycol. Recrystallization from ethyl acetate gave apparently contaminated crystals (mp 170.5 to 171° C) with a specific activity of 319 cpm per mg; the semicrystalline residue from the mother liquors counted 260 cpm per mg. The crystals, 7.1 mg, were treated to form the oxime (mp 208 to 210° C; 202 cpm per mg), which was further purified by chromatography (toluene/propylene glycol, 25 hours) and crystallized (mp 212 to 213.5° C). It counted 260 cpm per mg with a similar specific activity in the mother liquor residue. Despite the variations in specific activities, no evidence of radioimpurities was apparent. The androstenedione was considered pure at a specific activity of 275 cpm per mg.

Progesterone: The diluted substrate was recovered and recrystallized (acetone, mp 120 to 120.5° C). Its specific activity was 1,840 cpm per mg.

Radioautographs of the initial chromatograms revealed no zones of radioactivity other than those associated with the above compounds. The areas between progesterone and 4-androstenedione notably were free from radioactivity; the epimers 20α- and 20β-hydroxy-4-pregnen-3-ones, normal derivatives of progesterone in the ovary (34), have mobilities intermediate between those of the former compounds.

The data in Table IV for this Experiment B are calculated from the above specific activities and from the assumption that at the point of the second addition of carriers, 1.0 mg of each of the original steroid carriers was present.

EXPERIMENT C. Incubation of cholesterol-4-C^{14}: The mixture from this incubation was extracted in the usual way. The chloroform: methanol extract obtained at the outset was halved and to the separate portions, I and II, were added the steroid carriers listed in Table III. Each portion was treated as described above (Section III. *In vitro studies*); the phenolic fractions from portions I and II were combined.

EXPERIMENT C–I. The total ketonic material from portion I containing the added α,β-unsaturated ketosteroid carriers, weighed 122 mg and contained 80,000 cpm. It was separated on paper chromatograms in ligroin/propylene glycol as described in Experiments A and B.

11β-Hydroxytestosterone: This compound as isolated, was rechromatographed in toluene/propylene glycol, acetylated, and the 11β-hydroxytestosterone acetate chromatographed in ligroin/propylene glycol. The purified steroid was recrystallized several times (acetone: hexane) and finally melted at 127 to 128° C, and had a specific activity of 20 cpm per mg. The mother liquor residue counted 78 cpm per mg. The small amounts of substance precluded further manipulations and, therefore, definite radiochemical purity was not achieved.

11β-Hydroxyandrostenedione: This compound, following rechromatography in ligroin/propylene glycol (96 hours), was recrystallized from acetone: hexane to constant melting point (195 to 196° C) and a specific activity of 71 cpm per mg in the final crystals (8.0 mg).

Recrystallization from ethyl acetate: hexane gave crystals (5.6 mg, 69 cpm per mg) and an oily mother liquor residue (1.5 mg) counting 156 cpm per mg, suggesting that radiochemical purity had not been achieved.

17α-Hydroxyprogesterone: Following the separation of testosterone from this compound by the acetylation procedure, the 17α-hydroxyprogesterone was rechromatographed several times in toluene: methyl cyclohexane (50: 50)/propylene glycol and recrystallized from methanol to constant specific activity (50 cpm per mg) of both the final crystals and mother liquor residue.

Testosterone: Isolated as the acetate, this compound counted 25 cpm per mg. The mother liquor was not studied.

Androstenedione: Isolated and purified by rechromatography, androstenedione failed to crystallize. The oily product had a specific activity of 110 cpm per mg; this value is considered maximal.

Progesterone: This compound, purified as in Experiment A, was crystallized from acetone. The progesterone as crystals (mp 119 to 120° C) counted 193 cpm per mg; the progesterone as found in the last mother liquor counted 163 cpm per mg. The radioactivity in this carrier, as in all others, was considered too low to warrant further attention, in contrast to the activities in Experiments A, B and D.

EXPERIMENT C–II. 5-Androstenediol: The nonketonic fraction of portion II, containing the carrier 5-androstenediol and its associated radioactivity, weighed 29 mg and was chromatographed in toluene: methyl cyclohexane (50: 50)/propylene glycol for 48 hours. The androstenediol was located (antimony trichloride reagent), eluted and the chromatography repeated. Crystallization of the eluted steroid (acetone: hexane) yielded 7.3 mg of 5-androstenediol, mp to 175 to 177° C, specific activity 90 cpm per mg. After further addition of carrier (14.3 mg) to the above, the substance was acetylated and crystallized to give the diacetate, mp 157 to 158° C, specific activity 14 cpm per mg. Recrystallization from acetone gave crystals and a residue in the mother liquors counting, respectively, 13 and 17 cpm per mg.

3β,17α-Dihydroxy-5-pregnen-20-one: The ketonic fraction weighing 45.8 mg and containing 99,700 cpm, was dissolved in hot methanol and allowed to crystallize. Two crops of steroid were obtained, which were combined and recrystallized to constant melting point (226 to 236° C); the last crystals had a specific activity of 90 cpm per mg. Acetylation and crystallization of the latter from acetone gave 5.7 mg of 3β,17α-dihydroxy-5-pregnen-20-one acetate, mp 221 to 225° C, specific activity 93 cpm per mg and a mother liquor residue, 2.4 mg and 370 cpm per mg. Dilution of the crystals (4.0 mg) with carrier steroid acetate (4.0 mg) and recrystallization from acetone gave crystals (4.7 mg) with a specific activity of 52 cpm per mg, which did not change on recrystallization from methanol. The two mother liquor residues from these steps (1.3 mg each), however, counted 108 and 72 cpm per mg, respectively. Thus, despite apparent constant specific activity of the crystals, the mother liquors gave evidence of radiochemical contamination. Further purification was not possible because of the lack of material.

Dehydroepiandrosterone and 5-pregnenolone: The total ketonic fraction, less the crystalline 3β,17α-dihydroxy-5-pregnen-20-one, containing 28,000 cpm, was chromatographed on paper in methyl cyclohexane/propylene glycol for 41 hours. Two zones were visualized by the antimony trichloride reagent, eluted, and each was rechromatographed in the same solvent system.

The slower zone which gave a positive Zimmermann reaction was crystallized from hexane to give dehydroepiandrosterone (190 cpm per mg) which was diluted with an equal weight of carrier, acetylated and crystallized from hexane to give crystals counting 91 cpm per mg. This material was recrystallized and the crystals obtained (mp 168 to 169° C, corrected) counted 68 cpm per mg; the mother liquor residue counted 110 cpm per mg. Recrystallization of 2.0 mg of the crystals gave 0.8 mg of specific activity 74 cpm per mg and a new mother liquor residue (0.7 mg) counting 140 cpm per mg. Radiochemical purity could not, therefore, be demonstrated.

The faster zone was eluted, and after addition of carrier steroid (10.9 mg), was recrystallized to constant melting point (186 to 188° C) and a final specific activity of 208 cpm per mg; the mother liquor residues were at each step of higher specific activity. Acetylation and crystallization from methanol gave crystals of pregnenolone acetate (mp 148 to 149° C), whose specific activity was 125 cpm per mg; crystallized once more, the acetate counted 70 cpm per mg. The two last mother liquor residues counted 290 and 750 cpm per mg, respectively. The product was considered contaminated.

In contrast to Experiments A, B and D, little radioactivity was associated with the above steroid carriers at the point of their isolation from the chromatograms. This low radioactivity precluded the further addition of carrier-diluent and prevented use of recrystallization procedures for demonstrating and achieving radiochemical purity. The uncharacterized radioactivity present in the extracts was not associated with any chromatographically discrete compounds; it tended to smear throughout the paper chromatograms of both Experiments C-I and C-II. Whether this material is a product of a biochemical transformation cannot be inferred from this study; it may be entirely due to artifact formation caused by the manipulatory procedures. The values obtained in this study have not been included in the tabulation of the results from the other three studies (Table IV).

EXPERIMENT D. Incubation of acetate-1-C^{14}: The polar lipids [tubes 5 to 8 from the distribution between ligroin and methanol: water (90: 10)] were combined (2.62×10^8 cpm) and washed free of acetate-1-C^{14} by means of a 4-transfer ccd between water and chloroform, using the stripping procedure described by Weisiger (47). The upper phase of tube O was saturated aqueous sodium acetate (pH 7.0) instead of water. The resulting lipid fraction contained 2.68×10^6 cpm; apparently no appreciable amount of labeled acetate was present. This fraction was subjected to a phenolic separation (27), giving a

neutral fraction containing 2.20×10^6 cpm and a phenolic fraction (86,000 cpm) which was subjected to a ccd between toluene and 1 N sodium hydroxide, using five funnels and complete stripping. There was a peak in radioactivity which moved with the alkali and was extractable with ether at pH 8.5. The material in this peak, having the properties of phenols, contained 15,000 cpm. This purified phenolic fraction was subjected to a 99-transfer ccd in methanol: water (70: 30)/chloroform: carbon tetrachloride (40: 60). The distribution was analyzed for radioactivity and for the estrogens by the fluorimetric method. Estrone, estradiol-17β, and estriol were identified, but no peak in radioactivity was associated with any of the three estrogens. It was therefore concluded that none of the three was labeled.

Five mg each of 17α-hydroxyprogesterone, 11β-hydroxyprogesterone, 6β-hydroxyprogesterone and adrenosterone were added as additional carriers to the neutral fraction. This was subjected to a 150-transfer ccd in cyclohexane: ethyl acetate (50: 50)/ethanol: water (50: 50). The peaks of radioactivity ($K^2 = 0.32$ and $K = 1.02$) contained all of the neutral carrier steroids except progesterone and 5-pregnenolone which were found together in an area containing no peak of radioactivity ($K = 2.56$). It was, therefore, concluded that these latter two compounds were not appreciably labeled.

The contents of the tubes in each of the two peaks of radioactivity containing the steroid carriers were combined, resulting in a pool of polar ($K = 0.32$) material (Pool A) and one of less polar ($K = 1.02$) material (Pool B). Pool A contained all of the 11β-hydroxyandrostenedione and adrenosterone. Pool B contained all of the androstenedione, dehydroepiandrosterone, and 17α-hydroxyprogesterone; each pool contained part of the 6β-hydroxyprogesterone, 11β-hydroxyprogesterone and testosterone. Pool A was acetylated and chromatographed in the cyclohexane: toluene (50: 50)/propylene glycol system. Four zones were located on the chromatogram and eluted. These contained 11β-hydroxyandrostenedione, 11β-hydroxyprogesterone, adrenosterone, and a mixture of testosterone acetate and 6β-acetoxyprogesterone. This mixture of acetates was saponified with 1 N potassium hydroxide in methanol overnight at room temperature. The saponified material was chromatographed in the ligroin: toluene (67: 33)/methanol: water (70: 30) system. Three zones were located and eluted: testosterone, 6β-hydroxyprogesterone, and a less polar compound having the R_F of allopregnane-3,6,20-trione, the major product of saponification of 6β-acetoxyprogesterone with excess alkali (48).

Pool B was subjected to a digitonin separation (49). The "β-fraction" was chromatographed in the ligroin: toluene (67: 33)/methanol: water (70: 30) system to yield dehydroepiandrosterone. The "α-fraction" was acetylated and chromatographed in the ligroin/propylene glycol system. Three zones were located on the chromatogram and eluted; they were androstenedione, a mixture of 11β-hydroxyprogesterone and 17α-hydroxyprogesterone; and a mixture of the acetates of testosterone and 6β-hydroxyprogesterone. The mixture of 11β-hydroxyprogesterone and 17α-hydroxyprogesterone was resolved in the ligroin: toluene (67:33)/methanol: water (70: 30) system. The mixture of acetates of testosterone and 6β-hydroxyprogesterone was saponified and chromatographed in the same way as the corresponding fraction from Pool A. Again testosterone, 6β-hydroxyprogesterone, and allopregnane-3,6,20-trione were recovered.

At this point, all of the carriers from the two peaks of radioactivity in the 150-transfer ccd had been recovered chromatographically pure. In the cases in which the compound was recovered in both pools, the two portions were combined. Each carrier isolated was assayed for weight and radioactivity; additional unlabeled carrier was then added to each, and tests of radiochemical purity were applied. The details of these procedures are given for each compound.

11β-Hydroxyandrostenedione: Analysis by ultraviolet absorption at 240 mμ showed a recovery of 3.11 mg containing 109,000 cpm. An additional 50 mg of carrier was added and this was crystallized three times from aqueous ethanol yielding crystals having specific activities of 1,890, 1,850, and 1,880 cpm per mg (calculated, 2,050 cpm per mg) and a final mother liquor residue, specific activity 1,660 cpm per mg. Crystallization from benzene yielded crystals having a specific activity of 1,850 cpm per mg, and a mother liquor residue of 1,920 cpm per mg. The crystals from benezene were subjected to a 99-transfer ccd in methanol: water (70: 30)/chloroform: carbon tetrachloride (20: 80). The single peak in radioactivity coincided with the peak in material having ultraviolet (240 mμ) absorption ($K = 0.73$), and there was no evidence of lack of homogeneity (45). The mean specific activity in the region of the peak was $2,120 \pm 218$ (standard deviation) cpm per mg. 11β-Hydroxyandrostenedione was considered radiochemically pure at this point.

Adrenosterone: The adrenosterone isolated was measured by ultraviolet absorption (238 mμ), and contained 2.66 mg and 32,000 cpm. An additional 10 mg of unlabeled adrenosterone was added and a 99-transfer ccd was carried out using methanol: water (70: 30)/chloroform: carbon tetrachloride (10: 90). Analysis by ultraviolet absorption showed a single peak ($K = 0.62$), but radioactivity extended from tube O through the peak in adrenosterone, thereby demonstrating lack of purity. The contents of the tubes containing the adrenosterone were pooled and crystallized four times from aqueous ethanol, yielding crystals having specific activities at each step of 395, 300, 279, and 289 cpm per mg and a residue in the last mother liquor, specific activity of 308 cpm per mg. Crystallization from ethyl acetate: hexane gave crystals, specific activity 275 cpm per mg and a mother liquor residue of specific activity 264 cpm per mg. The final crystals and residue in the mother liquor were combined and subjected to a 49-transfer ccd in cyclohexane: ethyl acetate (60: 40)/ethanol: water (50: 50). The single peak in radioactivity coincided with the single peak in material absorbing at 238 mμ

[2] K = distribution coefficient.

(K = 0.71). The material was radiochemically pure as judged by the specific activity in the region of the peak which was constant at 277 ± 24 cpm per mg. The adrenosterone was considered radiochemically pure.

11β-Hydroxyprogesterone: The 11β-hydroxyprogesterone isolated was analyzed by ultraviolet absorption at 242 mμ, and consisted of 3.08 mg and 24,000 cpm; 10 mg of carrier was added. A 99-transfer ccd in methanol: water (70:30)/chloroform: carbon tetrachloride (20:80) showed a clear dissociation of radioactivity (K = 0.30) from the 11β-hydroxyprogesterone as measured by ultraviolet absorption (K = 0.41). Hence, the 11β-hydroxyprogesterone was not labeled.

6β-Hydroxyprogesterone: The portion of the 6β-hydroxyprogesterone carrier recovered unchanged was rechromatographed in ligroin: toluene (67:33)/methanol: water (70:30). The compound as isolated from this chromatogram was still not pure, as shown by an atypical ultraviolet absorption spectrum. The absorbance at 237 mμ indicated a maximum content of 0.13 mg; it contained 950 cpm. To this was added 8.6 mg of unlabeled carrier. A 99-transfer ccd in methanol: water (80:20)/chloroform: carbon tetrachloride (10:90) showed a clear dissociation of the radioactivity (K = 1.57) from the carrier (K = 1.41). The transformation product of 6β-hydroxyprogesterone, allopregnane-3,6,20-trione, as recovered from the chromatograms, contained only 3,700 cpm. This low level of radioactivity together with the lack of activity in the small amount of 6β-hydroxyprogesterone recovered made further work on this fraction seem unwarranted.

Androstenedione: The pool of this compound recovered from the incubation mixture contained 2.04 mg as measured by ultraviolet absorption at 240 mμ, and 133,000 cpm. To this was added 60 mg of unlabeled androstenedione. Two crystallizations from aqueous ethanol furnished crystals having specific activities of 1,980 and 1,900 cpm per mg (calculated 2,140 cpm per mg) and a final mother liquor, specific activity of 1,960 cpm per mg. Crystallization from acetone: cyclohexane yielded crystals of specific activity 1,880 cpm per mg and a mother liquor residue of 1,960 cpm per mg. These latter crystals were subjected to a 99-transfer ccd in methanol: water (80:20)/carbon tetrachloride. The single peak in radioactivity coincided with the peak in ultraviolet absorption (K = 0.72); the mean specific activity in the region of the peak was 2,130 ± 194 cpm per mg. The androstenedione was therefore considered radiochemically pure at this point.

17β-Hydroxyprogesterone: The pool of 17α-hydroxyprogesterone contained 1.61 mg (239 mμ) and 22,100 cpm; 10 mg of 17α-hydroxyprogesterone carrier was added and the whole subjected to a 99-transfer ccd in methanol: water (70:30)/chloroform: carbon tetrachloride (10:90). The peak in radioactivity coincided with the peak in the 17α-hydroxyprogesterone as measured by ultraviolet absorption (K = 0.49). The mean specific activity around the peak was 1,900 ± 167 cpm per mg. The material from this peak was crystallized twice from aqueous ethanol to give crystals having specific activities of 1,670 and 1,740 cpm per mg (calculated 1,900 cpm per mg). Crystallization from benzene: cyclohexane gave crystals, 1,740 cpm per mg and a mother liquor residue, 1,750 cpm per mg. The 17α-hydroxyprogesterone was considered radiochemically pure at this point.

Dehydroepiandrosterone: The recovered dehydroepiandrosterone was analyzed by the Zimmermann reaction (25) and contained 2.11 mg and 3,800 cpm; 10 mg of unlabeled dehydroepiandrosterone was added and the whole was subjected to a 99-transfer ccd in methanol: water (80:20)/carbon tetrachloride. A single peak (Zimmermann) of carrier was found (K = 1.25); there were two peaks in radioactivity—one (K = 8.00) contained 745 cpm; the larger peak corresponded to the peak in dehydroepiandrosterone (K = 1.25), but contained other labeled material since it failed to fit the theoretical distribution curve. This peak contained about 2,100 cpm. The contents of the tubes containing the dehydroepiandrosterone were combined and crystallized five times from aqueous ethanol giving crystals containing 87, 102, 86, 84, and 90 cpm per mg and a final mother liquor residue having 110 cpm per mg. Crystallization from acetone: hexane yielded crystals containing 85 cpm per mg and a mother liquor residue of 75 cpm per mg. The last crystals were subjected to a 24-transfer ccd in ethyl acetate: cyclohexane (30:70)/ethanol: water (55:45). The peak in radioactivity coincided with the peak in dehydroepiandrosterone (Zimmermann reaction, K = 1.04). Constant specific activity, 80 ± 12 cpm per mg in the region of the peak, indicated radiochemical purity. The dehydroepiandrosterone was then considered radiochemically pure.

Testosterone: The testosterone as isolated from the incubation mixture was impure as indicated by its ultraviolet absorption spectrum. It was, therefore, rechromatographed in ligroin: toluene (67:33)/methanol: water (70:30). It then had a satisfactory spectrum indicating a recovery of 1.12 mg and contained 4,800 cpm; 10 mg of carrier testosterone was added and the whole subjected to a 99-transfer ccd in methanol: water (80:20)/chloroform: carbon tetrachloride (10:90). A single peak in material absorbing at 240 mμ coincided with the single peak in radioactivity (K = 1.19); however, the experimental points for radioactivity did not agree well with the theoretical curve, thus indicating the presence of a radioactive impurity. The peak contained 9.73 mg and 3,380 cpm. The contents of the tubes containing the testosterone were crystallized five times from aqueous ethanol. The specific activities of the crystals were 306, 312, 255, 196, and 206 cpm per mg, and that of the residue in the final mother liquor, 276 cpm per mg. Crystallization from acetone: hexane yielded crystals containing 223 cpm per mg and a residue in the mother liquor of 210 cpm per mg. In view of these results and the relatively high level of activity associated with the closely related androstenedione, the testosterone was considered labeled.

DISCUSSION

Methods for radiochemical purity

In the demonstration of radiochemical purity of the isolated steroid carriers, it has been our experience that radioactive contamination is relatively unlikely when the radioactive starting material is a steroid closely related to the products; thus, in Experiments A and B, achievement of radiochemical purity presented no serious difficulties. This was not true when the radioactive starting material was a substance far removed (in the biosynthetic sense) from the products under study, as in Experiments C and D in which cholesterol-C^{14} and acetate-C^{14} were incubated. In one, only a small percentage of the radioactivity was ultimately associated with the steroid carriers, while in the other (Experiment C), demonstration of radiochemical purity was not achieved; the purification problem in these latter cases appears to have been compounded by the large variety and unknown nature of the radioactive impurities, particularly in the cholesterol-C^{14} study.

We have found that after separation and isolation of the carrier steroids in *chemically* pure state, several purification measures may be used to demonstrate radiochemical purity, the main criterion being constancy of specific activity of the steroid at each step through a series of physical and chemical manipulations. The greater the number of such manipulations without significant change in specific activity, the greater the likelihood that the compound is radiochemically pure. Conversely, decreases in specific activity through these manipulations signify contamination of the carrier by some trace impurity.

Among the various manipulations currently employed for purification, countercurrent distributions have proven particularly useful, since *nonhomogeneity* can be readily detected when the radioactive compound and the steroid carrier have only slightly different partition coefficients in the system employed (45). Formation of suitable derivatives is also of value, since two compounds (steroid carrier and radioactive compound) which have very similar physical properties may form derivatives having less similar properties. In this regard, the steroid carrier derivative selected should be one which is based upon the most characteristic chemical feature of the carrier and which effects the maximum alteration in its physical properties. Crystallization (of steroid carriers and derivatives), however, has been the manipulation most widely used as the criterion for radiochemical purity, since a trace contaminant is likely to be completely soluble in the solvent used to crystallize the larger amount of carrier. Unfortunately, with steroids, this ideal situation is often complicated by adsorption of impurities on crystals and even mixed crystal formation (this phenomenon may explain the tenacity of the contaminants encountered in Experiment C). Purification to constant specific activity in a particular solvent may be considered to have been achieved when specific activity remains constant through several recrystallizations. When only small quantities of carrier are available, however, the above is not always possible; in such cases we have found it necessary to carry out specific activity measurements on *both* crystals and mother liquors from each crystallization in order to determine if constant specific activity has been achieved in that particular solvent.

These criteria served to provide the values given in Table IV for Experiments A, B and D; the same criteria applied to the steroids in the cholesterol-C^{14} study (Experiment C) indicated that the associated radioactivity was largely due to unknown impurities. To be noted is the value of the isolation of several carriers devoid of radioactivity; such negative results lend considerable confidence to the positive values obtained in the same study.

Biochemical considerations

The elevated level of the urinary 17-ketosteroids seen in the patient preoperatively is consistent with the classical clinical features of interstitial cell tumors of the testis in childhood (1–21). Analysis of the individual 17-ketosteroids revealed androsterone and etiocholanolone (Table II) to be present in the largest amounts. Metabolic studies in humans (50) show that the principal precursors of these two urinary $C_{19}O_2$ steroids are androstenedione, testosterone and dehydroepiandrosterone, and that the two metabolites are usually excreted in approximately equal amounts, as they are in our patient (Table II). The elevated levels of the 11-oxygenated 17-ketosteroids in the

urine are most impressive since the occurrence of an oxygen substituent at carbon-11 of a steroid of mammalian origin has long been considered indicative of that steroid's adrenal cortical origin. The high concentration of 11-keto- and 11β-hydroxyandrosterone (1.8 mg per 24 hours) indicates that at least one of the likely precursors (51)—11β-hydroxyandrostenedione, adrenosterone or 11β-hydroxytestosterone—was formed by the tumor; these three latter compounds have been isolated from adrenal tissue either as constituents or as metabolic products (52–55). The urinary 17-ketosteroid pattern thus suggests that the principal $C_{19}O_2$ androgen elaborated was testosterone or androstenedione (or perhaps dehydroepiandrosterone), and that one of the more weakly androgenic $C_{19}O_3$ compounds—11β-hydroxyandrostenedione, 11β-hydroxytestosterone, or adrenosterone (56, 57)—was formed in lesser amounts. The preoperative levels of urinary pregnanetriol suggest also the likelihood of the formation by the tumor of 17α-hydroxyprogesterone (50).

The results of the acetate incubation study, given in Table IV, indicate that androstenedione and testosterone are indeed the major steroids elaborated by the tissue under the conditions of incubation and, no doubt, account for the virilizing properties of this tumor. The accumulation of C^{14} in both these compounds amounts to 0.07 per cent of the added acetate. 11-β-Hydroxyandrostenedione and its companion adrenosterone (11β-hydroxytestosterone was not sought) accumulated 0.04 per cent. Thus, in the acetate experiment, the ratio of $C_{19}O_2$ to $C_{19}O_3$ steroids is in excellent agreement with the quantitative relationship of these two classes of steroids found in the urine.

In the study with acetate-C^{14}, little or no radioactivity was associated with progesterone while a significant amount accumulated in 17α-hydroxyprogesterone. Perfused stallion testes have been shown to incorporate acetate-C^{14} into both progesterone and 17α-hydroxyprogesterone (58), and a human embryonal testicular tumor, under tissue-slice conditions, elaborated progesterone (31) in addition to androstenedione and testosterone. Our incubation studies suggest that while these two C_{21}-compounds appear to act in this tumor as intermediates (see below) in the biosynthetic pathway to the androgens (Experiment B), both may well have been secreted by this tumor *in vivo*, their production being reflected by the elevated urinary titers of pregnanetriol and neutral acylable lipids (Table I), although the latter may be made up of C_{19}-diol compounds. The relatively minor amount of dehydroepiandrosterone obtained in the incubation with acetate-C^{14} (Table IV), suggests that this substance was transformed rapidly by the tissue and therefore accumulated little or no radioactivity in the *in vivo* study.

The incubation of the tumor tissue with cholesterol-C^{14} was undertaken with the notion that an efficient utilization and transformation of labeled cholesterol into steroid hormone products would occur. This premise has proved to be false. This does not necessarily mean that cholesterol is not an intermediate in the synthesis of steroid hormones in this tumor. Although the tissue was capable of active steroid synthesis as seen in the other experiments, no significant amount of cholesterol-C^{14} was transformed into recognizable C_{19} or C_{21} steroid products. Dilution of the radioactive cholesterol by the tissue and medium cholesterol, or its failure to penetrate cell membranes could explain the negative results. However, the cholesterol-C^{14} did undergo some chemical transformations to more polar material, as indicated by the large amounts of radioactivity found in the polar fractions accompanying the steroid carriers. This unidentified material, which may have been a biochemical transformation product or an artifact, accompanied the carrier steroids through several purifications, including the formation of derivatives.

The results of the incubation of testosterone-C^{14} (Experiment A) are clear-cut. The major transformation product of testosterone was androstenedione, known to be a normal secretory product of human (59) and dog testicular tissue (60). The presence in this tumor of the enzyme, 11β-hydroxylase (61), was demonstrated by the isolation of radioactive 11β-hydroxyandrostenedione and 11β-hydroxytestosterone (54). The significance of this reaction in testicular tumor tissue is discussed below. The formation of radioactive adrenosterone in minor amounts points to the presence of the widely occurring 11-hydroxydehydrogenase (62) in this tumor tissue or possibly in the plasma used in the incubating medium. It

should be noted that no radioactive products other than the four described were found in this experiment and that the 11β-hydroxylase activity of the tissue is demonstrated by the fact that 7.6 per cent of the added testosterone was converted to 11-oxygenated steroids.

The isolation of radioactive 17α-hydroxyprogesterone, testosterone and androstenedione from the progesterone-C^{14} incubation experiment (Table IV, Experiment B) serves to indicate the pathway for the formation of androgens by this tumor tissue. Slaunwhite and Samuels (63) described the transformation of progesterone into androstenedione and testosterone by rat testicular tissue and postulated that 17α-hydroxyprogesterone is an intermediate. These transformations have been confirmed by the present study, by Solomon, Vande Wiele and Lieberman (64) in bovine ovarian tissue, and by Lynn and Brown (65) and one of the authors in normal rat testis tissue, which has also been shown to transform 17α-hydroxyprogesterone-C^{14} into radioactive androstenedione and testosterone (66). The presence of 11β-hydroxyandrostenedione among the transformation products of progesterone is consistent with the results of the testosterone incubation. The absence of 11β-hydroxyprogesterone and 11-ketoprogesterone from the products derived from progesterone is interesting in view of the isolation of 11-oxygenated C_{19} compounds in this and other incubations. This may suggest that the tumor 11β-hydroxylase exhibits a substrate preference (61). A final point of interest is the apparent failure of this tumor tissue to convert progesterone to either of the two substances, 20α- or 20β-hydroxy-4-pregnen-3-one. They have been shown to be produced from progesterone by human (66) and bovine (67) ovarian tissue *in vitro*, and by the eviscerated rat *in vivo* (68), and normally accompany progesterone in the blood of pregnant women and in human follicles, corpora lutea and possibly in placental blood (35).

Clinical considerations

There are 22 previously reported cases of so-called interstitial cell tumors of the testis occurring in childhood and producing isosexual precocity (1–21). In the present case the demonstration in the tumor of certain biochemical activities (11β-hydroxylase activity, formation of 11β-hydroxyandrostenedione and related compounds), which more closely resemble those of adrenal cortical than of testicular tissue, requires reappraisal of the status of the interstitial cell tumor of the testis.

As with the previous 22 cases, our patient displayed clinical abnormalities attributable to the overproduction of androgens: precocious statural and isosexual growth, advanced skeletal maturation, excessive muscular development, facial acne and hirsutism. Precocity began between the ages of three and six years in almost all reported cases, including the present one, and the tumor was unilateral and benign in all but one case. Correlated with this, in our case and nine previous cases so studied, urinary 17-ketosteroids were elevated for the age, ranging from 2.9 to 64 mg per 24 hours. Gynecomastia was observed in three previous cases but not in the present one, where urinary estrogen was in the normal range and none was formed by the tumor tissue *in vitro* (Experiment D). A clue to the gynecomastia in the three cases may be in the recent demonstration of the conversion of androgen to estrogen by human endocrine (69) and nonendocrine tissues (70) and the observation of gynecomastia following androgen administration (71).

The origins of these tumors are not clear. First, there are no precise morphologic criteria for differentiating adrenal cortical cells from interstitial cells of Leydig (11, 72), and secondly, adrenal cortical rests do occur in the testes. This is not surprising since the adrenal cortex arises embryologically in the dorsal mesenteric root in close proximity to the urogenital ridge containing the gonads (73). In this way it is conceivable that these tumors can resemble adrenal cortical tissue both morphologically and biochemically. On the other hand, massive replacement of testicular tissue by aberrant adrenal cortical cells in a patient with congenital virilizing adrenal cortical hyperplasia has been reported by Wilkins and co-workers (74). Subsequently, this has been confirmed, and in 1954, Hedinger reported a similar patient in whom cortisone administration induced marked regression of testicular size (75). Staubitz and co-workers in 1953 reported a patient with bilateral "interstitial cell tumors" with precocity beginning at three years of age, but in whom urinary

17-ketosteroids were 73.9 mg per 24 hours before bilateral orchiectomy and still elevated at 69.6 mg per 24 hours three months postoperatively (76), suggesting that this was an instance of adrenogenitalism with bilateral adrenal cortical rests in the testes; for this reason it has not been included in our list of previously reported cases of interstitial cell tumors of the testis. Because of insufficient data for diagnosis, another case with bilateral testicular tumors reported by Garvey and Daniel has been excluded from our series (77). We have accepted the case reported by Rezek and Hardin (19) with bilateral testicular tumors, primarily on the basis of a decrease in 17-ketosteroids from 91 (60 per cent "β-ketosteroids") to 2.1 (no "β-ketosteroids") mg per 24 hours 16 months postoperatively. However, the preoperative level of 3β-hydroxy-17-ketosteroids in the urine is suggestive evidence for tumor formation from adrenal-cortical cell rests. Sandblom's patient has been included perhaps incorrectly in our series, since at four years of age and one year postoperatively the urinary 17-ketosteroids varied between 10 and 20 mg per 24 hours as compared to 64 mg per 24 hours before surgery (8). There thus appears, probably for want of completeness in their work-up, an obscure picture of the "testicular" origins of these tumors; in some instances they possess the distinct character of adrenal cortical rests, while in those included in our series, they show no such morphological properties.

There is one histological finding that has long been considered specific for Leydig cells, namely the presence of crystalloids of Reinke (78). These also occur in the ovarian hilus cells but have never been described in adrenal cortical tissue. Recently Sternberg, Segaloff and Gaskill have reaffirmed that these crystalloids are indeed "specific identification tags, distinguishing hilus and Leydig cells from morphologically similar cells such as lutein and adrenal cortical cells" (79). Although absent in each of the previously reported cases of prepubertal interstitial cell tumors, crystalloids of Reinke were clearly demonstrated in the tumor of our patient. Thus, on the one hand this tumor had this morphologically identifying stigma of testicular origin, while on the other hand, its biochemical activity suggested certain adrenal cortical properties. Here the enigma must rest, but we are forced to re-open the question as to tissue specificity of both the crystalloids of Reinke and the capacity to elaborate 11β-hydroxysteroids. Perhaps it should be re-emphasized that while 11β-hydroxylase activity has never been previously observed in normal testicular tissue, no distinct search has been made until very recently; it was not found in normal stallion testis tissue (58) and could not be demonstrated in an arrhenoblastoma (66). The apparent absence of 11β-hydroxylase from normal testicular and ovarian tissues may however reflect a failure of analytical techniques to detect extremely low concentrations of enzymatic activity. The process of tumor formation could bring about an increase in concentration of an enzyme normally considered absent from the tissue, and seemingly could cause the tumor to "acquire" an enzyme not usually associated with the parent tissue. The recent report of the enzyme 21-hydroxylase (likewise generally associated with adrenal cortical tissue) in an *induced* tumor of testicular interstitial cell origin in the mouse (80) suggests such a possibility.

Our patient's postoperative course during two years of observation has been of considerable interest. His excessive libido gradually subsided and social adjustment improved, but there was no regression of the physical manifestations of precocity; indeed, he continued to mature. Growth continued at approximately the same rate as preoperatively, but the bone age advanced only one to two years. The remaining left testis, small preoperatively, increased to late-adolescent size with normal consistency. Urinary gonadotropins, which were absent preoperatively, reached the normal adult range of 7 rat uterine units. The urinary 17-ketosteroid levels fell precipitously after operation to approximately 1.0 mg per 24 hours, but during the last year of observation have varied between 3.0 and 7.4 mg per 24 hours. The above data suggest that postoperatively the patient entered true puberty with its attendant gonadotropin production, growth spurt and maturation of the remaining testis. This simulates the sexual maturation which has been observed during cortisone therapy of patients with the adrenogentital syndrome in whom somatic maturation, reflected by a bone age of 11 to 12 years, had taken place prior to treatment (81). It would appear that, irrespective of chronological age, some re-

lationship exists between the level of somatic and skeletal development and the initiation of the hypothalamic-pituitary discharge which leads to elaboration of gonadotropin. The normal adolescent values of urinary gonadotropins, 17-ketosteroids and pregnanetriol postoperatively in our patient are evidence against either development of a new tumor in the remaining testis or of adrenal hyperplasia as a basis for the subsequent continued somatic and sexual development.

Inadequate data are available on the postoperative course of the 22 previously reported cases. In general, the literature reports little or no regression of the precocious physical characteristics. In two patients with preoperative heights of 152 cm there was no further growth postoperatively, probably reflecting closure of the epiphyses prior to surgical excision of the tumors. However, in five patients whose preoperative height was less than 127 cm and whose bone age was 12 years or less, a deceleration of growth rate and genital development was specifically noted. One other patient, with a preoperative height of 120.7 cm and a bone age of 10 years, grew 20 cm during the first 17 months after operation, and the remaining testis appeared to be enlarging. While the latter patient's course is suggestively similar to that of our own case, no testicular biopsy nor gonadotropin assays were reported. In three patients with gynecomastia, breast enlargement disappeared in two and persisted in one.

This protracted study has served to demonstrate that the virilizing properties of this tumor may be ascribed to two principal androgenic steroids, testosterone and 4-androstenedione, whose formation in the tumor is consistent with the mechanism of androgen biosynthesis recently postulated for normal testicular tissue (63) (Figure 7). The demonstration of the presence of the enzyme 11β-hydroxylase points to certain hitherto unsuspected endocrine and embryological considerations of this tumor. The implications of these observations as to the nature of the hormone-secreting cells of this tissue must await the extension of these studies to similar virilizing tumors of the testis.

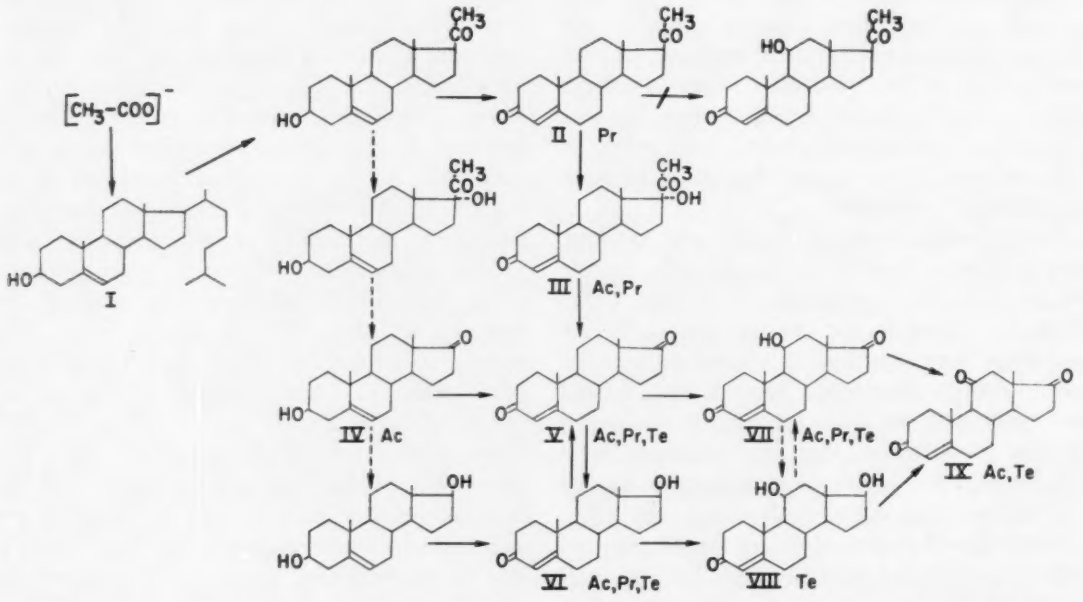

FIG. 7. PROBABLE PATHWAY OF STEROID BIOSYNTHESIS IN THE TESTICULAR TUMOR. Broken arrows indicate presumed transformations; Te = produced from testosterone-4-C^{14} in Experiment A; Pr = produced from progesterone-4-C^{14} in Experiment B; Ac = produced from acetate-1-C^{14} in Experiment D. I = cholesterol; II = progesterone; III = 17α-hydroxyprogesterone; IV = dehydroepiandrosterone; V = 4-androstene-3,17-dione; VI = testosterone; VII = 11β-hydroxy-4-androstene-3,17-dione; VIII = 11β,17β-dihydroxy-4-androstene-3-one (11β-hydroxytestosterone); IX = adrenosterone.

SUMMARY

An interstitial cell tumor in the testis was removed from a highly virilized boy. The preoperative levels of urinary 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, 11-ketoandrosterone, androsterone, etiocholanolone and pregnanetriol were abnormally high and fell promptly following surgery. After incubation of tumor slices with acetate-1-C^{14}, the following radioactive steroids were found by carrier dilution: testosterone, 4-androstene-3,17-dione, (in small amount) dehydroepiandrosterone, 17α-hydroxyprogesterone, adrenosterone and 11β-hydroxyandrostenedione; estrone, estradiol-17β, estriol, progesterone and 11β- and 6β-hydroxyprogesterone were isolated devoid of radioactivity. Incubation with progesterone-4-C^{14} yielded radioactive 17α-hydroxyprogesterone, androstenedione, testosterone and 11β-hydroxyandrostenedione. Incubation with testosterone-4-C^{14} yielded radioactive androstenedione, 11β-hydroxytestosterone, 11β-hydroxyandrostenedione and adrenosterone. Cholesterol-4-C^{14} failed to give rise to significant amounts of radioactive steroid products.

A critique of methods for the study of radiochemical purity of steroids is presented.

The morphological, biochemical and clinical aspects of this twenty-third reported case of virilizing interstitial cell tumor of the testis are discussed.

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DISAPPEARANCE RATE OF CONCENTRATED PROCONVERTIN EXTRACTS IN CONGENITAL AND ACQUIRED HYPOPROCONVERTINEMIA *

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While developing methods for extraction and concentration of various adsorbable blood clotting factors (1–3), we found that an extract made by $Al(OH)_3$ adsorption of citrated plasma contained large amounts of proconvertin and only small quantities of prothrombin, Stuart factor (4), and plasma thromboplastin component (PTC) (5). The present report is concerned with the disappearance rate of proconvertin following the rapid intravenous administration of this extract to nine patients with congenital or acquired hypoproconvertinemia.

In their original description of congenital proconvertin [1] deficiency, Alexander, Goldstein, Landwehr and Cook (6) indicated that the corrective effect of normal plasma or serum transfusion was lost within 24 hours. After transfusion of whole blood to an infant with severe congenital hypoproconvertinemia, Hitzig and Zollinger (7) found the proconvertin half-life to be 115 to 135 minutes. The half-life was 57 minutes following injection of 20 ml of an extract having a proconvertin potency 30 times that of normal plasma.

Frick (8) stated that the half-life of proconvertin was 5.5 to 6 hours following plasma transfusion in normal subjects made hypoproconvertinemic by the intravenous administration of Warfarin sodium.[2] Didisheim, Loeb, Blatrix and Soulier (9) stated that the half-life of proconvertin was 50 to 55 hours, following intravenous administration of a concentrated proconvertin extract to one patient with cirrhosis of the liver.

All previous investigators have been able to demonstrate exponential disappearance curves with only a single component. We have repeatedly found exponential curves with two components in all experiments except that performed on the normal subject. Both the net rise in proconvertin in the blood and the disappearance rate following injection of the extract were dependent on the degree of deficiency in the recipient.

METHODS

All blood was drawn in uncoated glass syringes, immediately mixed with 1/10 volume of 3.2 per cent sodium citrate in 0.7 per cent NaCl and centrifuged at 2,000 rpm for 20 minutes. The plasma was removed and stored in stoppered glass tubes at $-20°$ C.

Proconvertin. This was measured by a modification of the method of Owren and Aas (10), using plasma from two patients (Bi. C. and Bo. C.) with severe congenital proconvertin deficiency as substrate. These patients had normal prothrombin consumption and thromboplastin generation tests and normal concentrations of Stuart factor. Almost identical results were obtained using partially adsorbed beef plasma as substrate in tests on patients having normal Stuart factor concentrations. The proconvertin time was determined after the addition of 0.1 ml 0.025 M $CaCl_2$ to a mixture of 0.1 ml of substrate plasma, 0.1 ml rabbit brain thromboplastin, and 0.1 ml of the patients' plasma (1/10 dilution in 0.85 per cent NaCl). Duplicate tests were done on each sample and the times averaged. The proconvertin concentration was determined by referring to a standardization curve which was constructed separately for each new lot of reagents. All tests in any given experiment were performed on the same day with the same reagents. The standardization curve was constructed by plotting the proconvertin time against the plasma dilution on log-log paper using 1/10 to 1/200 dilutions of normal plasma. The results obtained in 5 to 7 normal subjects were averaged. Our normal value for this test is 100 per cent ($2SD = \pm 25$ per cent). Deficiencies were arbitrarily classified as being *mild* with values between 50 and 75

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[1] Serum prothrombin conversion accelerator (S.P.C.A.), Factor VII, stable factor (SF), stable prothrombin conversion factor, co-thromboplastin.

[2] Warfarin is 3-(α-acetonyl benzyl)-4-hydroxy-coumadin. The sodium salt is produced under the name of Coumadin Sodium by Endo Products Inc., Richmond Hill, N. Y.

per cent, *moderate* between 20 and 49 per cent and *severe* between 0 and 19 per cent.

Prothrombin complex and Stuart factor. Prothrombin complex activity was measured by the method of Quick (11) using dehydrated rabbit brain as the source of tissue thromboplastin. Prothrombin and Stuart factor were measured by the method of Hjort, Rapaport and Owren (12). Stuart factor was measured by the method of Bachmann, Duckert and Koller (13), which is similar to the method of Sise, Lavelle and Becker (14) except that optimum concentrations of cephalin and Russell viper venom are determined.

Preparation of extract. Citrated (ACD) blood bank plasma, from 1 to 26 days old, was stored in the frozen state until sufficient quantity had been accumulated for extraction (3 to 4 months).[3] The plasma was thawed at 37° C to a final temperature of 25° C and mixed with $Al(OH)_3$ (assayed as 2.4 per cent Al_2O_3 in water at neutral pH) in 1/75 of the original plasma volume for 30 minutes. After centrifugation the $Al(OH)_3$ sediment was washed with 0.9 per cent NaCl in 3/4 of the original plasma volume for 30 minutes at 25° C and centrifuged. The washed sediment was eluted with 0.08 M Na_2HPO_4 solution, adjusted to pH 8.0 in 1/4 of the original plasma volume, stirred for 30 minutes at 25° C and centrifuged. The supernatant was decanted and brought to pH 5.6 in a concentration of 40 per cent ethanol at −5° C, suspended in cold water and freeze-dried. The dried precipitate was dissolved in 1/100 original plasma volume of Plasmanate,[4] sterile filtered and stored in the frozen state or lyophilized. The material was tested in rats and rabbits for toxicity and pyrogenicity before use in human subjects.

RESULTS

Clotting factor content

Three lots of extract prepared from 25 to 100 L pools of plasma were used. The content of clotting factors as compared with normal plasma was: proconvertin, 35 to 88 times; prothrombin plus Stuart factor, 6 to 30 times; Stuart factor, 3.6 to 8.6 times; proaccelerin, antihemophilic factor and thrombin absent; and PTC negligible.

Stability

The lyophilized extracts were stable when tested at 18 months, the reconstituted frozen extract at 17 months, and the reconstituted liquid extract after storage at room temperature for 48 hours. The proconvertin potency of the extract was not diminished after incubation with the plasma of a patient with severe congenital hypoproconvertinemia for 140 minutes at 37° C.

Administration

Sixteen studies were made on one moderately deficient and four severely deficient patients with congenital hypoproconvertinemia, two patients with mild deficiency of proconvertin of unknown etiology, one patient with severe hepatitis, one normal subject and the same subject made hypoproconvertinemic by the oral administration of Warfarin. A summary of the results is given in Table I. In these experiments 14 to 58.5 ml of extract was given intravenously within three to seven minutes. Because of the small volumes employed no correction for plasma volume expansion was made. The plasma volume was not measured and an average value of 45 ml per kg was used in calculating the predicted net rise in proconvertin at T = 0 (time of completion of injection). For purposes of these experiments one unit of proconvertin is defined as the amount in 1 ml of normal human plasma.

Urine recovery experiments

In several experiments no proconvertin could be recovered in the urine collected during the period following injections of the extract when 90 per cent of the proconvertin activity disappeared from the blood. The volume of urine collected during this time was only 10 per cent of the plasma volume so that if any proconvertin was present it should have been easily detected. This does not prove, however, that proconvertin was not lost through the kidney and subsequently inactivated.

Reactions

Except for transient flushing of the face in two patients during the injection there were no subjective reactions during or following the use of these extracts. The temperature, pulse and respiration remained unchanged. The normal subject, a physician, subsequently developed acute hepatitis at a time consistent with the incubation period of homologous serum jaundice. However,

[3] We are indebted to the Irwin Memorial Blood Bank of the San Francisco Medical Society for its cooperation in collecting these plasmas.

[4] Produced by the Cutter Laboratories, Berkeley, Calif. The product is made from the effluent of Cohn fraction IV-1 and contains approximately 88 per cent albumin, the remainder being α- and β-globulins. It is heated for 10 hours at 60° C to destroy the virus of homologous serum jaundice.

TABLE I

Disappearance rate of concentrated proconvertin extracts after intravenous injection

Case	Diagnosis	Age	Sex	Experiment no.	Preinjection proconvertin concentration	Proconvertin potency of extract in multiples of normal plasma	Amount injected	Amount injected	Net rise at T = 0	Predicted net rise at T = 0	Per cent recovery	Half-time of exponential components	
												1st	2nd
		yrs			*% of normal*		*ml*	*units*/kg*	*% of normal*	*% of normal†*		*min*	*min*
Bi. C.	Severe congenital hypoproconvertinemia	6	M	1	3	58.0	14	36.3	96	80	120	12	90
				2	7	60.0	28	70.0	160	177	90	15	100
Bo. C.	Severe congenital hypoproconvertinemia	8	M	2	3	57.0	22	44.8	102	100	102	15	95
				1	6	85.5	20	61.0	132	135	98	17	100
				3	2	69.2	21	48.4	96	107	90	14	95
M. R.‡	Severe congenital hypoproconvertinemia	11	F	1	1	51.1	28	44.6	91	99	92	12	110
N. C.§	Severe congenital hypoproconvertinemia	23	M	1	4	59.5	56	42.0	96	93	103	11	110
M. M.	Moderate congenital hypoproconvertinemia	35	M	1	26	55.0	45	36.0	118	80	147	28	225
				2	32	70.0	40	41.0	136	91	149	30	240
				3	35	88.0	25	32.3	110	72	152	30	222
Mc. C.	Mild hyproconvertinemia	62	M	1	74	85.5	30	34.2	145	76	180	18	210
G. L.	Mild hypoproconvertinemia	27	M	1	70	40.8	44	25.6	93	57	162	25	213
V. W.	Acute toxic hepatitis	40	F	1	5	45.0	42	37.8	101	84	117	15	120
P. A.	Normal	47	M	1	102	35.86	58.5	32.9	122	73	167		230
P. A.	Normal	47	M	1	13	44.3	28	19.2	46	43	106	12	85
	Receiving Warfarin			2	14	42.2	52	34.3	95	76	125	13	85

* For purposes of these experiments, one unit of proconvertin is defined as the amount in 1 ml of normal plasma.
† Based on an average plasma volume of 45 ml per kg.
‡ We are indebted to Dr. Louis K. Diamond for permission to study this patient at the Children's Hospital, Boston, Mass.
§ We are indebted to Dr. Samuel Rapaport for permission to study this patient at the University of California Hospital at Los Angeles.

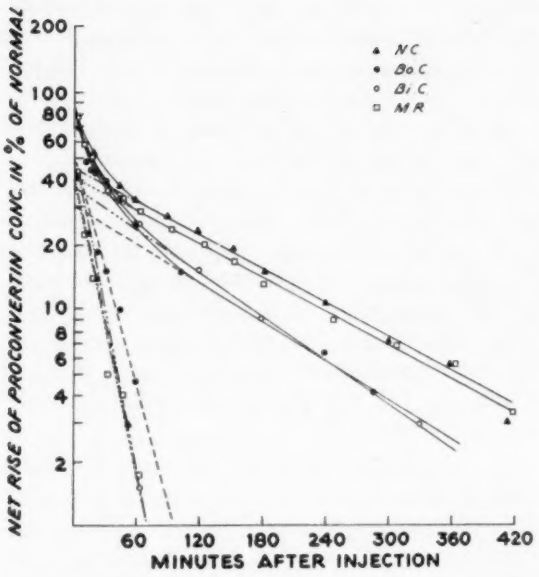

FIG. 1. DISAPPEARANCE CURVES FOLLOWING INJECTION OF CONCENTRATED PROCONVERTIN EXTRACT IN FOUR PATIENTS WITH SEVERE CONGENITAL HYPOPROCONVERTINEMIA. (N. C., 42.0 U per kg; Bo. C., 48.4 U per kg; Bi. C., 36.3 U per kg; M. R., 44.6 U per kg.)

at the time he received the extracts, he was also exposed to several cases of infectious hepatitis. None of the other five patients who received the same lot of extract, nor those receiving other lots, developed any late sequelae.

Analysis of disappearance curves.

Disappearance curves having two exponential components were obtained in all experiments except the one in the normal subject. The net rise in proconvertin at $T = 0$ was obtained by extrapolation of the exponentials. The two exponential components (net rise of proconvertin concentration $= Ae^{-k_1t} + Be^{-k_2t}$) were determined graphically by subtraction of the extrapolated final slope (Be^{-k_2t}) from the initial portion of the curve; Ae^{-k_1t} was plotted in this way. The net rise in proconvertin at $T = 0$ is equal to the sum of the extrapolated exponential component intercepts ($A + B$) (15).

A. Severe congenital hypoproconvertinemia. In Figure 1 are shown the results of a single experiment in each of four patients with severe congenital hypoproconvertinemia (N. C., M. R., Bo. C. no. 3 and Bi. C. no. 1). The results of three experiments done on one of these patients (Bo. C.) are shown in Figure 2. The similarity of the slopes of the disappearance curves is apparent. In seven experiments done on these four patients, the half-time of the first exponential component of the disappearance curve varied from 11 to 17 minutes and that of the second from 90 to 110 minutes. The dose of proconvertin varied from 36.3 to 70 units per kg. Within this range the slope of the disappearance curve did not appear to be dose dependent. The net rise in proconvertin in the patient's blood at $T = 0$ was 91 to 160 per cent; the predicted net rise was 80 to 177 per cent. The net recovery averaged 99 per cent of the predicted value.

B. Moderate congenital hypoproconvertinemia. In one patient (M. M.) with moderate congenital hypoproconvertinemia considerably slower disappearance rates were found than in patients with the severe form of the disease. In Figure 3 one experiment on this patient (M. M. no. 3) is compared with a similar experiment on a patient (Bo. C. no. 2) with severe congenital hypopro-

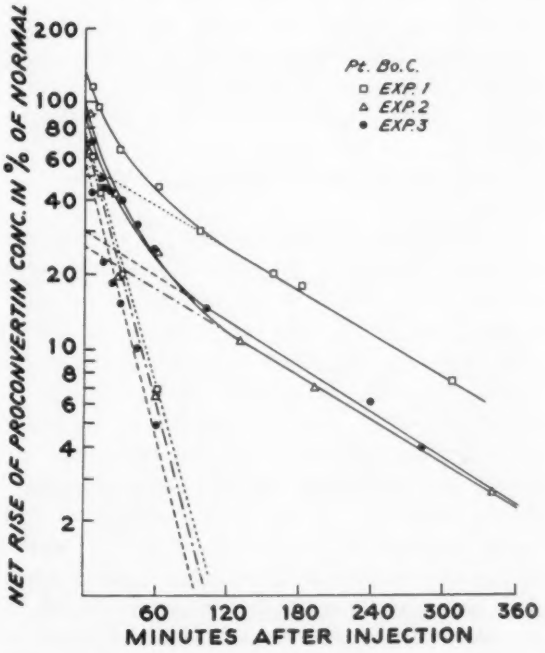

FIG. 2. DISAPPEARANCE CURVES FOLLOWING INJECTION OF CONCENTRATED PROCONVERTIN EXTRACT ON THREE OCCASIONS TO ONE PATIENT WITH SEVERE CONGENITAL HYPOPROCONVERTINEMIA. (Bo. C.: Exp. no. 1, 61.0 U per kg; Exp. no. 2, 44.8 U per kg; Exp. no. 3, 48.4 U per kg.)

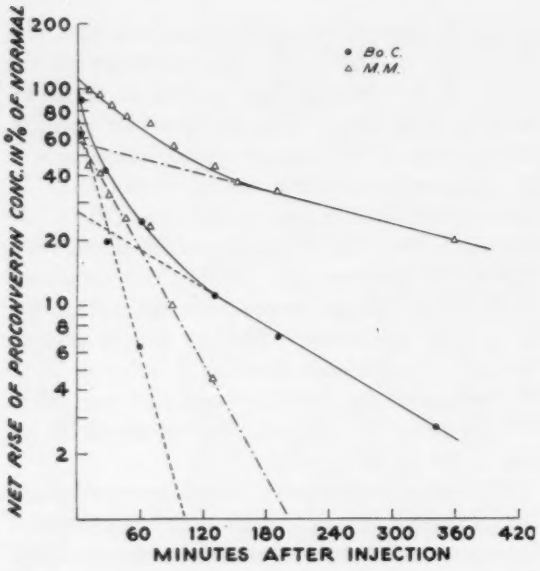

FIG. 3. DISAPPEARANCE CURVES FOLLOWING INJECTION OF CONCENTRATED PROCONVERTIN EXTRACT TO ONE PATIENT WITH SEVERE (Bo. C., 44.8 U PER KG) AND ONE WITH MODERATE (M. M., 32.3 U PER KG) CONGENITAL HYPOPROCONVERTINEMIA.

convertinemia. Following doses of 32.3 to 41 units per kg, the half-time of the first exponential component of the disappearance curve was 28 to 30 minutes and that of the second 222 to 240 minutes. The net rise in proconvertin in the patient's blood at T = 0 was 110 to 136 per cent. The net recovery was 147 to 152 per cent of the predicted value.

C. Mild hypoproconvertinemia. Two patients with mild fluctuating hypoproconvertinemia were studied. One, Mc. C., is the maternal grandfather of Bi. C. and Bo. C. He had no history of abnormal bleeding. At various times his proconvertin concentration had been found to be between 54 and 70 per cent. The other patient, G. L., had a mild bleeding tendency, his most recent episode having been prolonged bleeding following hemorrhoidectomy. His proconvertin concentration had varied between 18 and 74 per cent. The results of the experiments in these patients, done when their proconvertin concentrations were 70 and 74 per cent, were in most respects similar to those in the patient with moderate congenital hypoproconvertinemia. The half-time of the first exponential component of the disappearance curve was 18 and 21 minutes while that of the second was 210 minutes in both cases. Following doses of 25.6 and 34.2 units per kg the net rise in proconvertin in the patient's blood at T = 0 was 93 and 145 per cent, which amounted to 162 and 180 per cent of the predicted value.

D. Liver disease. The results of one experiment performed in a patient with acute hepatitis with a proconvertin concentration of 5 per cent were similar to those obtained in the patients with severe congenital hypoproconvertinemia. The half-time of the first exponential component was 15 minutes and that of the second 120 minutes. Following a dose of 37.8 units per kg, the net rise of proconvertin in the patient's blood at T = 0 was 101 per cent which amounted to 117 per cent of the predicted value.

E. Normal subject before and after Warfarin. One normal subject was studied before and after the oral administration of Warfarin. The disappearance curves following administration of the extract are shown in Figure 4. Before Warfarin a disappearance curve was obtained with a single exponential component, with a half-time of 230 minutes. The net rise of 122 per cent in proconvertin at T = 0 in the patient's blood, after injection of 32.9 units per kg, was 167 per cent of the predicted value.

Over a period of 12 days he was given orally

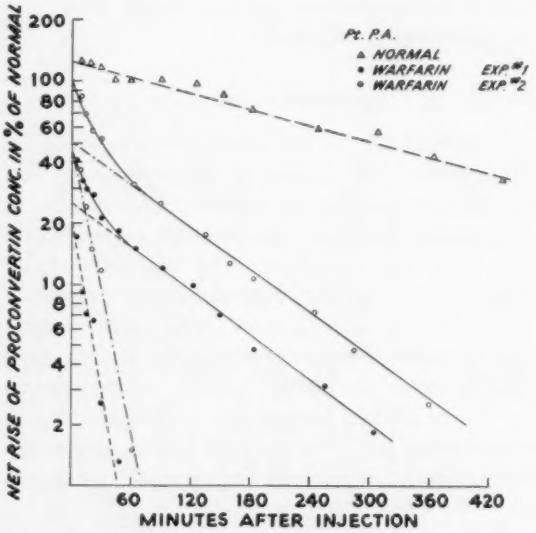

FIG. 4. DISAPPEARANCE CURVES FOLLOWING INJECTION OF CONCENTRATED PROCONVERTIN EXTRACT TO A NORMAL SUBJECT BEFORE AND AFTER THE ADMINISTRATION OF WARFARIN. (Normal, before, 32.9 U per kg; after Warfarin, Exp. no. 1, 19.2 U per kg; after Warfarin, Exp. no. 2, 34.3 U per kg.)

12.5 mg Warfarin daily until his proconvertin level had become stabilized. This dose was continued throughout the period of the experiments. During the five days preceeding the first experiment his proconvertin concentration varied between 9 and 13 per cent, and the Quick test and the Stuart factor concentrations varied between 18 and 22 per cent. In the first experiment he was given a dose of extract sufficient to produce a net rise of 46 per cent in the proconvertin concentration of his blood at T = 0. Two days later he was given approximately twice this dose which resulted in a net rise of 96 per cent at T = 0. The net recoveries were 106 and 125 per cent of the predicted values.

The slopes of the disappearance curves were almost identical in the two experiments, the half-time being 12 and 13 minutes for the first component and 85 minutes for the second. These results are similar to those found in severe congenital hypoproconvertinemia and are in striking contrast to those obtained in the same subject befor the administration of Warfarin.

Effect of extracts on other clotting tests

The effect of the extracts on clotting factors other than proconvertin was studied in several patients. Three of the patients with untreated

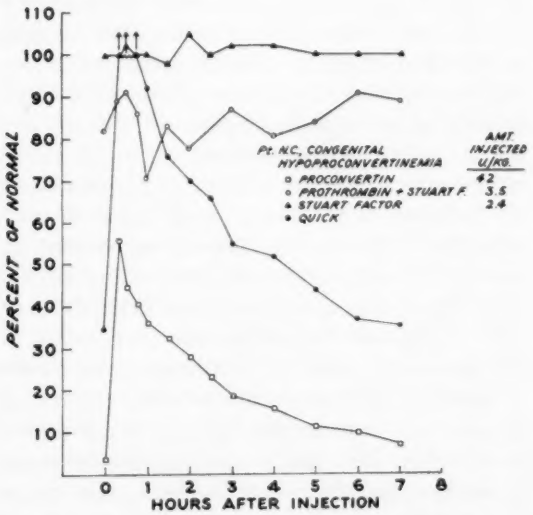

FIG. 5. THE EFFECT OF THE ADMINISTRATION OF A CONCENTRATED PROCONVERTIN EXTRACT ON THE QUICK, PROCONVERTIN, PROTHROMBIN AND STUART FACTOR TESTS IN A PATIENT WITH SEVERE CONGENITAL HYPOPROCONVERTINEMIA.

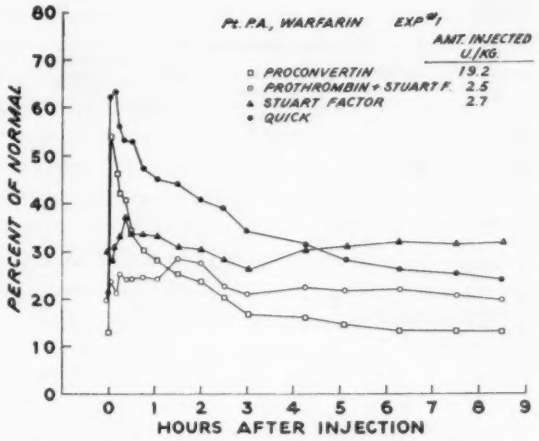

FIG. 6. THE EFFECT OF THE ADMINISTRATION OF A CONCENTRATED PROCONVERTIN EXTRACT (19.2 U PER KG) ON THE QUICK, PROCONVERTIN, PROTHROMBIN AND STUART FACTOR TESTS IN A NORMAL SUBJECT RECEIVING WARFARIN.

severe congenital deficiencies (Bi. C., Bo. C. and M. R.) with proconvertin concentrations of 1 to 7 per cent had Quick test values of 9 to 12.5 per cent. One patient (N. C.) showed a greater discrepancy, his Quick value being 36 per cent with a proconvertin concentration of only 4 per cent. In all of these patients following injection of the extract the Quick values remained higher than the proconvertin concentration, particularly during the first hour when differences of 35 to 60 per cent were frequently noted. This increased discrepancy gradually diminished toward the end of the experiments and was always gone by the following day. A comparison of the results of these two tests in N. C. following injection of the extract is shown in Figure 5.

In the one patient with *moderate congenital hypoproconvertinemia* there was fairly good agreement between the results of the Quick and proconvertin tests both before and after all three injections of extract. Before injection the proconvertin varied between 26 and 35 per cent and the Quick value between 36 and 38 per cent. Following injection the Quick value was usually slightly greater than the proconvertin concentration but there were variations of as great as ± 10 per cent.

In the *normal subject receiving Warfarin* two different situations were observed immediately following injection of the extract, depending upon the amount given. With the smaller dose, which elevated the proconvertin concentration to 54 per

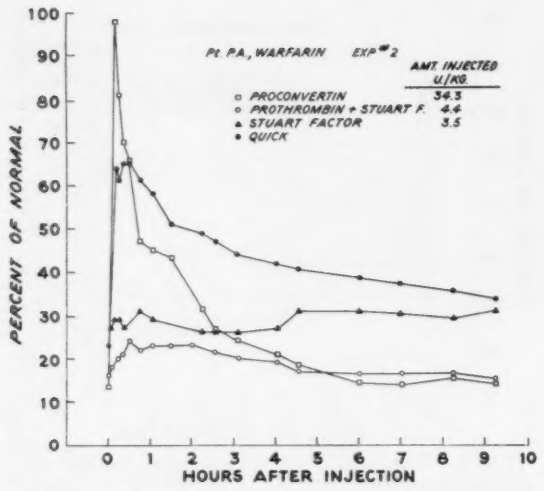

FIG. 7. THE EFFECT OF THE ADMINISTRATION OF A CONCENTRATED PROCONVERTIN EXTRACT (34.3 U PER KG) ON THE QUICK, PROCONVERTIN, PROTHROMBIN AND STUART FACTOR TESTS IN A NORMAL SUBJECT RECEIVING WARFARIN.

cent ten minutes after injection, the Quick value exceeded the proconvertin value by 9 per cent and remained 11 to 19 per cent higher throughout the period of the experiment (Figure 6). With the larger dose of extract, which elevated the proconvertin concentration to 96 per cent, the proconvertin value exceeded the Quick value by 35 per cent ten minutes after injection. At 30 minutes the results of the two tests were equal. Thereafter the Quick value exceeded the proconvertin result by as much as 29 per cent (Figure 7). These results show the strong influence which can be exerted on the Quick test by variations in the proconvertin concentration. Up to a certain point, despite a considerable deficiency in prothrombin and Stuart factor, elevation of the proconvertin concentration was capable of producing a similar elevation in the results of the Quick test. When the proconvertin concentration was raised to still higher levels, however, the Quick value did not increase proportionately. In this case the critical point was only slightly below the lower limit of normal for the Quick test. The effect of a normal proconvertin concentration in masking a deficiency of prothrombin or Stuart factor is apparent.

DISCUSSION

There was good agreement between the observed and predicted net increase in proconvertin concentration at $T = 0$ following the injection of extract in the severely deficient patients. The observed net increase, however, was consistently higher than that predicted in the normal and less severely deficient patients. This difference was not related to age, sex, body weight or the condition responsible for the proconvertin deficiency. It did not appear to be caused by the actual level of proconvertin measured in the patient's blood since in one severely deficient patient a total concentration of 167 per cent was achieved, while in several experiments on mildly and moderately deficient patients the highest concentration measured was below this level. Several different interpretations of these results are possible but none is convincing. Technical laboratory error seems unlikely because of the smoothness of the curves and the consistency of the finding. A systematic error in the method, resulting in values which were too low when proconvertin was measured in the extracts *in vitro* or too high when it was measured in the blood of the patient after administration of the extract *in vivo*, could account for the discrepancy.

In all experiments except that performed on the normal subject, disappearance curves having two exponential components were obtained. Both components of the curve had a faster rate in the more severely deficient patients, regardless of whether the cause of the deficiency was congenital or secondary to liver disease or the administration of Warfarin. It is not possible to determine from our data whether the first exponential represents endothelial adsorption, transfer of proconvertin into an extravascular compartment, a change *in vitro* in the degree of activation of proconvertin, the influence of a cofactor or inhibitor, or some other mechanism. The second exponential appears to measure metabolic destruction since it proceeds at a steady rate to an infinitely small value. Because the mechanism responsible for the first exponential of the disappearance curve is unknown, it is impossible to state why its rate is faster in the severe deficiencies. However, the data derived from the second exponential appear to indicate that the metabolic destruction rate of proconvertin is more rapid the more severe the deficiency. These results suggest that the reduced proconvertin concentration in these conditions may actually be caused by an increase in its rate of

destruction as well as by a decreased rate of production.

The *apparent* disappearance rate of a substance is influenced by its rate both of production and destruction. We have made the fundamental assumption that all of the patients in this study were in a steady state at the time when our experiments were performed. We found no evidence that the rate of production of proconvertin is controlled by its concentration in the blood since there was no correlation between the total proconvertin concentration achieved and its apparent disappearance rate after injection of extract. If the production rate were controlled by the blood level, one would have expected the normal subject, whose proconvertin concentration was raised to 224 per cent, to have experienced a decrease in production rate and therefore to have shown the fastest *apparent* disappearance rate of the entire group, whereas, in fact, his was one of the slowest. Conversely, while the same subject was receiving Warfarin, a small dose of extract produced an elevation of his proconvertin concentration to a level of only 59 per cent, yet in this experiment the disappearance rate was faster than in any other experiment in the series. However, it is possible that Warfarin, liver disease, and the congenital deficiency state raise special considerations and that in the normal subject in the steady state the production rate of proconvertin is controlled by its level in the blood.

The site and mechanism of destruction of proconvertin are unknown. It does not seem probable that it is normally consumed in a process of continuous *in vivo* coagulation. If so, its rate of utilization should depend on the availability of other clotting factors. However, in the Warfarin experiments, prothrombin, Stuart factor, and probably PTC were markedly reduced, yet the disappearance rate of proconvertin was as rapid as in the severe congenital deficiencies in which these factors were present in normal concentrations. Furthermore, proconvertin is not required for the production of blood thromboplastin and is not destroyed in the process of blood clotting.

One hypothesis which might explain some of these results would involve the assumption that a part of the proconvertin in the body exists in an extravascular or endothelial compartment. In the severe deficiencies less proconvertin would be present in this compartment and it might, therefore, accept more proconvertin at a faster rate. Conversely, in the less deficient patients the compartment would be more nearly saturated and might be less capable of accepting additional proconvertin. The half-life of the first component of the disappearance curve would be shorter in the former instance than in the latter. The disappearance curve having a single exponential component in the normal subject might be explained on the assumption that the compartment was completely saturated, or at least had a higher concentration of proconvertin than that in the circulating blood.

The fact that the rates of both components of the disappearance curve show a direct relationship to each other suggests that the mechanism for the initial removal of injected proconvertin from the circulating blood may be the same as that for its ultimate destruction. A mechanism involving a greater degree of cellular or membrane permeability might account for the faster disappearance rate of both components of the curve in the severe deficiency states. An increased perfusion rate could result in more rapid delivery of proconvertin to a specific organ of destruction and also to tissues where it is not destroyed and can be fed back to the circulating blood. In this regard, it is of interest to note that Dicumarol significantly increases the initial transvascular transfer of T-1824-labeled plasma protein in the rabbit (16).

The differences between our results and those reported by Hitzig and Zollinger (7) in an infant with severe congenital hypoproconvertinemia are not great and could well be due to differences in the materials and techniques employed.

There are several reasons for the discrepancy between Frick's (8) results and ours in the normal subject receiving Warfarin. Following the administration of approximately 1,000 cc of plasma in 30 minutes to four normal adult subjects, he reported a disappearance curve with a single exponential component having a half-time of 330 to 360 minutes. The net increase in clotting factor value was 18 to 35 per cent. We produced net increases in proconvertin of 46 and 95 per cent following the administration of concentrated extract within 5.5 minutes to one normal subject receiving Warfarin, and in both instances ob-

tained disappearance curves having half-times of 12 and 13 minutes for the first exponential and 85 minutes for the second.

We have re-evaluated Frick's data and find that when the *net* increase in proconvertin over the baseline value (rather than total concentration) is plotted on semilog paper, the half-time shown by his data is 145 to 165 minutes rather than 330 to 360 minutes. Furthermore, the test he employed did not specifically measure proconvertin but rather a complex composed of proconvertin and Stuart factor. Since the half-time of Stuart factor appears to be longer than that of proconvertin, it is probable that the disappearance rate of the proconvertin was actually very close to that which we obtained. His failure to observe an initial more rapid component in the disappearance curve could have been due to the relatively long time required for the plasma infusion. Equilibrium could already have been established by the time his first observations were made 10 minutes after completion and 40 minutes after starting the plasma infusion.

In reporting a single experiment in a patient with cirrhosis of the liver, Didisheim and co-workers (9) stated that following injection of an extract containing approximately equal amounts of proconvertin and Stuart factor the half-time of proconvertin during equilibrium was 50 to 55 hours. Their data do not support this conclusion, since they also used a test which did not specificially measure proconvertin but rather the proconvertin, Stuart factor complex. Furthermore, at three hours after injection the result of this test had returned to its preinjection level. The further decrease in the test value over the subsequent four days could have been caused by the slower disappearance rate of Stuart factor contained in the extract given in the above experiment and on the previous day.

Because of the limitations of large plasma infusions it is difficult to compare their effects with those of concentrated extracts. Under the best of circumstances it is not possible to raise the proconvertin concentration in the recipient by more than 35 to 40 per cent with plasma infusions, and in interpreting the results one must consider the additional variable of changes in plasma volume. However, if allowances are made for these factors, Frick's results with plasma do not differ greatly from those we have obtained with concentrated extracts. Further studies with both substances will be required to determine whether the proconvertin in the extracts behaves in any way different from that in native plasma.

An opportunity for clinical trial of these extracts was provided by two patients with congenital hypoproconvertinemia. In one patient (M. M. no. 3) gingival bleeding of 24 hours' duration, following scaling of his teeth, immediately stopped after injection of 25 ml of extract. On three occasions severe epistaxis of 12 to 24 hours' duration was promptly controlled in Bi. C. by the intravenous administration of 15 to 30 ml of extract. In each of the above episodes active bleeding was in progress at the time of injection and was observed to stop within five minutes after its completion. No further bleeding occurred and complete healing ensued. On another occasion Bi. C. had frequent bloody stools of five days' duration, resulting in a decrease in his hemoglobin concentration to 4.2 g. Following administration of 28 ml of extract, a single tarry stool occurred but no further gross bleeding was observed. The net increase in proconvertin at $T = 0$ was 80 per cent. On all of the above occasions the amount of extract administered was sufficient to raise the patient's proconvertin concentration to or above the normal level. Although this effect was transient, the results suggest that if complete normality of the clotting mechanism can be achieved for even a short time it may be sufficient for normal hemostasis and healing to occur. However, since elevation of the Quick test value was of greater degree and longer duration than that of the proconvertin concentration, the possibility must be entertained that the therapeutic effects may have been due, at least in part, to some other unidentified prothrombin conversion accelerator contained in the extracts.

In all of the known blood clotting factor deficiency states except hypoproconvertinemia there is a defect in the intrinsic production of blood thromboplastin. In Stuart factor deficiency and hypoproaccelerinemia there is an additional failure of the extrinsic clotting mechanism which requires tissue thromboplastin for its proper functioning. It is only in hypoproconvertinemia that the coagulation defect is strictly confined to the extrinsic mechanism. It is therefore possible that

the cessation of bleeding following the use of extracts which produce such transient restoration to normality may be uniquely associated with proconvertin deficiency states. Similar trials with large doses of concentrated extracts of other clotting factors in the appropriate deficiency states will be required to clarify this point.

SUMMARY

Extracts containing 35 to 88 times the proconvertin concentration of normal human plasma were produced by aluminum hydroxide adsorption of citrated plasma, elution with phosphate buffer, and precipitation of the protein in the eluate at pH 5.6 with 40 per cent ethanol at $-5°$ C. Following 16 injections of these extracts into nine human subjects, net increases in proconvertin of from 46 to 160 per cent were achieved. In one normal subject a disappearance curve with a single exponential component having a half-time of 230 minutes was found. In two mildly deficient and one moderately deficient congenital hypoproconvertinemic patients disappearance curves with two exponential components were found. The half-time of the first component was 18 to 30 minutes and that of the second was 210 to 240 minutes. In four severely deficient congenital hypoproconvertinemic patients, one patient with severe liver disease, and one normal subject receiving oral Warfarin, disappearance curves with two exponential components were found. The half-time of the first component was 11 to 17 minutes and that of the second 85 to 120 minutes.

The average net increase in proconvertin at $T = 0$ was 104 per cent of the predicted value in the severely deficient patients and 159 per cent in the normal and mildly and moderately deficient patients.

Intravenous administration of the extract on five occasions to two patients with congenital hypoproconvertinemia resulted in prompt cessation of gingival bleeding, epistaxis and rectal bleeding.

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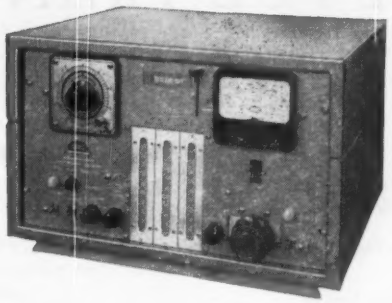

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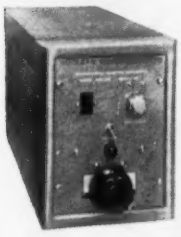

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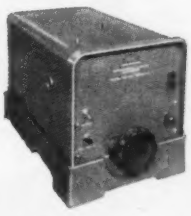

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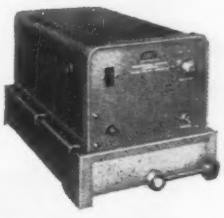

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Original Article

Action Inhibitrice d'une Hormone Oestrogène sur la Consommation Spontanée d'Alcool par le Rat. P. ASCHKENASY-LELU.

Éffets de l'Irradiation x Totale sur les Principaux Constituants Chimiques du Foie de Cobaye. O. LARTIGUE.

Interréaction des Plaquettes et du Plasma dans la Phase Initiale de la Coagulation. Technique d'Etude chez le Sujet Normal du Rôle des Plaquettes. Éffet du Contact. J. CAEN et FR. HAAS.

Étude Clinique et Physiopathogénique de la "Dystrophie d'Albright" (Pseudo-Pseudo-Hypoparathyroïdisme et de Syndromes Voisins. A Propos de Trois cas Nouveaux, Dont Deux Familiaux. CH. NAGANT DE DEUXCHAISNES, G. ISAAC, A. JACQUET et J.-J. HOET.

Original Notes

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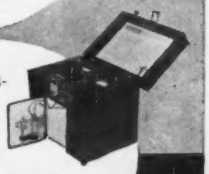

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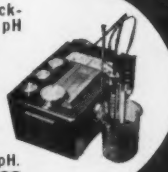

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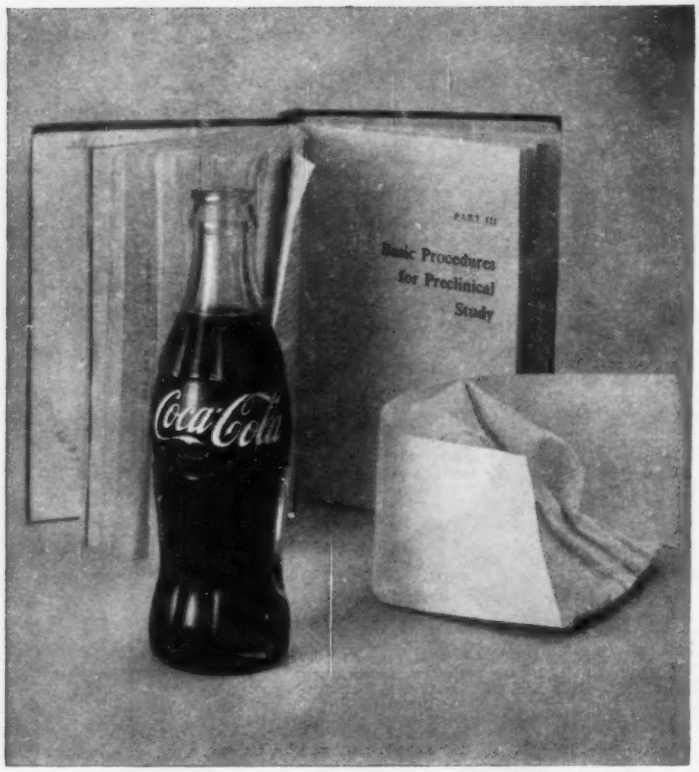

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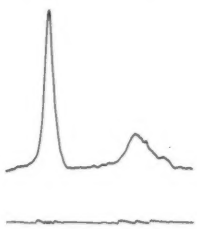

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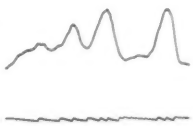

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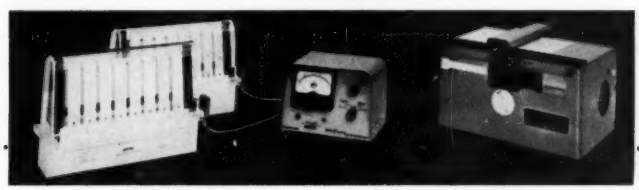

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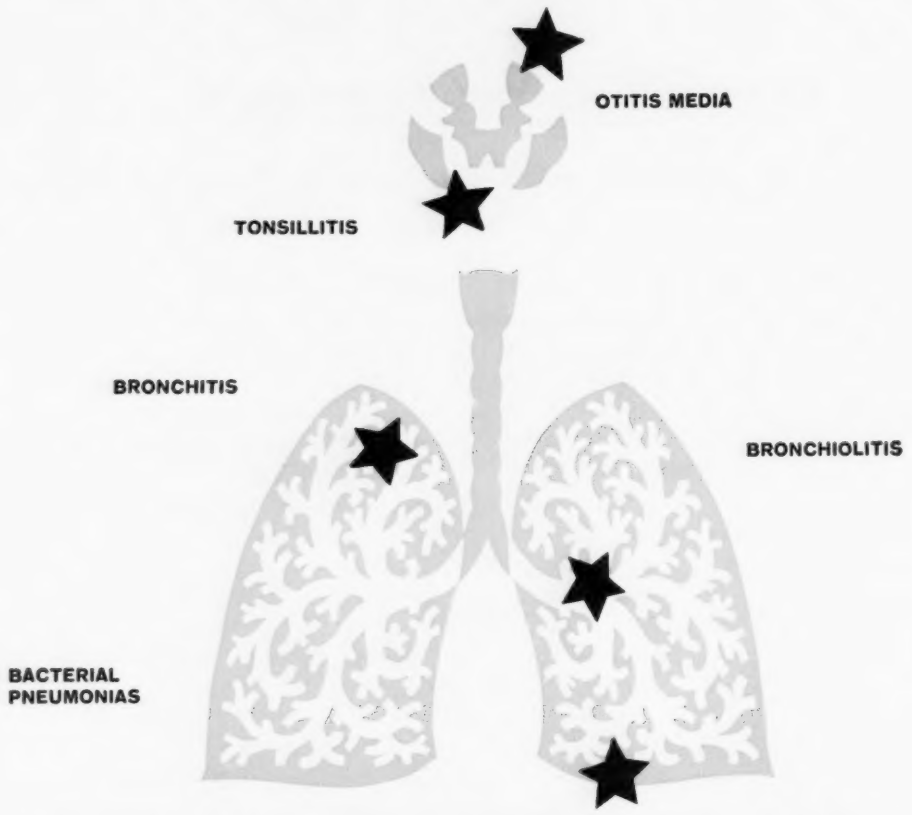

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